AUG 14 1925

Volume XX August, 1925 Number 2

# SOIL SCIENCE

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PUBLISHED MONTHLY
THE WILLIAMS & WILKINS COMPANY
MT. ROYAL AND GUILFORD AVENUES
BALTIMORE, MARYLAND, U. S. A.

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# THE NODULE BACTERIA OF SOYBEANS: I. BACTERIOLOGY OF STRAINS[1]

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Received for publication March 27, 1925

As early as 1895, Nobbe and his co-workers (33) studied the adaptability of strains of legume bacteria from different genera of legume plants, and came to the conclusion that the bacteria in the nodules of all legumes were strains of the same organism.

The investigations of Hiltner and Stormer (24) led them to classify the nodule bacteria into two groups. Theirs was largely a morphological classification.

More recently Burrill and Hansen (6), by a very extensive series of cross-inoculations, demonstrated eleven groups of the legume bacteria. When arranged according to cultural characters, all of the cultures in the eleven groups could be placed in only three groups.

The nodule bacteria from fourteen different legumes were carefully studied by Löhnis and Hansen (30). These investigators found it possible to divide their 57 cultures into two groups, differing morphologically as well as physiologically.

The accumulated data at the present time indicate that there are at least two morphological types of the legume bacteria, viz., small rods with single flagella, and small rods with several peritrichous flagella. These two types show many differences within themselves, not only of special adaptation to certain host plants, but varying physiological properties of different strains for the same group of host plants.

There is a wide variation in the effect of inoculation on the growth of soybeans on different soils as observed under Wisconsin conditions. The published researches concerning the nutrition of legumes show that many factors are concerned. Chief among these are physical composition of the soil, moisture, temperature, aeration, chemical composition of the soil solution, variety of plant and extent of its inoculation. Obviously, many of the factors which affect the green plant will also affect the nodule bacteria.

The extensive distribution of the legume bacteria as well as their host plants, the *Leguminosae*, indicates a wide range of adaptability of these microörganisms.

[1] Part I of a thesis submitted to the Faculty of the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Published with the approval of the Director of the Agricultural Experiment Station, Madison, Wisconsin.

The great similarity of the nodule bacteria and their resemblance to other soil bacteria have made necessary plant inoculation as the only reliable means of identifying them. The differences that have been observed in groups of closely related pathogenic bacteria suggest the possibility of similar differences within the groups of the legume bacteria.

The results reported in this paper include a morphological, serological, cultural, and physiological study of eight strains of *Pseudomonas radicicola* (Beijerinck) of *Soja max.*

## MORPHOLOGY OF CULTURES

### *History of cultures*

The original experiments were started in 1921 with six strains of bacteria. Later (1923) two more were added. A brief history of the cultures is given below.

| STRAIN | ORIGIN | NUMBER OF ANNUAL TESTS |
|---|---|---|
| | | *years* |
| 1 | N. Y. College of Agriculture, 1919 | 5 |
| 2 | Bureau of Plant Industry, U. S. Dept. Agr., 1918 | 5 |
| 3 | Madison, Wis, 1920 | 4 |
| 4 | University of Illinois, 1921 | 3 |
| 5 | U. S. Dept. Agr., 1921 | 4 |
| 6 | U. S. Dept. Agr., 1921 | 4 |
| 7 | Isolated from commercial culture, 1922 | 2 |
| 8 | Isolated from commercial culture, 1920 | 4 |

During the progress of the work two formulas for agar were tried. In the beginning, regular mannitol agar was used. This medium has the following composition:

| | *gm.* |
|---|---|
| Mannitol ($C_6H_8(OH)_6$) | 15.0 |
| Sodium chloride (NaCl) | 0.2 |
| Di-basic potassium phosphate ($K_2HPO_4$) | 0.2 |
| Magnesium sulfate ($MgSO_4$) | 0.2 |
| Calcium sulfate ($CaSO_4 \cdot 2H_2O$) | 0.1 |
| Calcium carbonate ($CaCO_3$) | 5.0 |
| Distilled water | 1,000.0 |
| Washed agar | 15.0 |

Reaction adjusted to pH 6.8.

At times considerable difficulty was experienced in getting some strains to grow well on this medium. This was especially true of strains 5 and 6. In order to secure greater and more rapid growth of all strains for inoculation purposes the medium was modified until better growth was secured. The modified medium contained only 10 gm. of mannitol and 1 gm. of calcium

carbonate. The phosphate was increased to 0.5 gm. and 100 cc. of sterile yeast water was added to the solution. The yeast water was prepared according to the method of Fred, Peterson and Davenport (19).

The purity of the cultures was repeatedly confirmed by plant passage, and by microscopic and cultural tests. The cultural characteristics of the legume bacteria as described by Löhnis and Hansen (30) were found useful in checking the purity of the cultures. The cultures did not contain *Bacillus radiobacter* (*B. radiobacter*, Beijerinck) at the start and were free from this organism more than four years later, after several plant passages.

### *Cultural characters*

The cultural characters of the strains were studied in the following media: mannitol yeast-water agar slant, meat-infusion agar slant, meat-infusion gelatin stab, meat-infusion broth, citrated milk, litmus milk and potato. All

TABLE 1

*Differences in cultural characters of soybean bacteria*

2 weeks at 28°C.

| STRAIN | YEAST-WATER MANNITOL AGAR SLANTS | MEAT INFUSION BROTH | POTATO |
|---|---|---|---|
| 1 | Moderate, white, dense, glistening growth | No growth | No growth |
| 2 | Moderate, white, dense, glistening growth | No growth | No growth |
| 3 | Moderate, white, dense, glistening growth | No growth | No growth |
| 7 | Moderate, white, dense, glistening growth | No growth | No growth |
| 4 | Moderate, white to translucent growth | No growth | No growth |
| 8 | Moderate, white to translucent growth | No growth | Very slight growth |
| 5 | Transparent, thin growth | Growth | Thin growth |
| 6 | Transparent, thin growth | Growth | Thin growth |

media except the litmus milk and potato were adjusted to a reaction of pH 6.8.

*Test tube cultures.* A summary of the most important cultural characters is shown in table 1. A comparison of the cultural characters of the eight strains shows some notable differences. All strains grew best on the yeast-water mannitol agar, and grew well in citrated milk but showed no differences in this medium. Litmus milk was turned alkaline by all strains, but more alkaline by strains 1, 2, 3, 4 and 7 than by strains 5, 6 and 8. The cultures on meat-infusion agar and gelatin did not grow enough to be of any cultural value. These media are not suited to any of the strains. Two of the eight strains, 5 and 6, grew well in meat-infusion broth. The others did not grow in this medium.

The great difference in appearance of the cultures is shown by the mannitol yeast-water agar slants. All cultures except 5 and 6 tended to produce moderate to abundant, translucent to white streaks, which were raised and glisten-

ing. Cultures over two weeks old frequently developed white colonies in the streaks especially at the bottoms of the tubes near the water of condensation. Cultures 5 and 6, on the other hand, produced thin, transparent films tending to spread. The mass of growth seldom became very dense, nor did it collect in white confluent colonies. Often the growth was so transparent it could be seen only by means of reflected light. All strains gave characteristic growth on yeast-water mannitol agar in one week at 28°C. The characters were much more pronounced in two weeks.

It has been stated that a medium of sterilized potato may be used for tests of cultures of legume bacteria because they do not grow on this medium, while common contaminants and *B. radiobacter* grow well on it. This is undoubtedly true for the usual incubation period of five days to one week at 28°C. However, when some of the strains are left on potato for four weeks or more, growth is apparent and may be confirmed by microscopic examination. Strains 5 and 6 gave the most growth on potato. Preparations from these cultures revealed typical bacteroids (plate 1, fig. 1). None of the cultures had ever been observed to show bacteroid formation in soybean nodules.

The variation in the type of growth produced by the two groups may be due to a difference in slime production. During the progress of the work, the growth from agar streaks was often mixed with distilled water and 0.85 per cent salt solution for the preparation of suspensions. It was repeatedly noticed that strains 5 and 6 quickly formed uniform suspensions. On the other hand, the other strains would give suspended clumps which were broken up only with severe and prolonged shaking. Although the mass of growth of most of the strains was tenacious, none of them showed the viscosity of alfalfa or pea organisms.

A comparison of the cultural characters of the eight strains showed some distinct differences. From table 1 it can be seen that the cultural characters gave three groups among the eight strains. Strains 1, 2, 3, 4 and 7 comprise one group. These grew well on yeast-water mannitol agar, but did not grow in meat-infusion broth or on potato. Strains 5 and 6 on the other hand produced a thin transparent type of growth on yeast-water mannitol agar, grew in meat-infusion broth and to a slight extent on potato. Culture 8 seemed to be somewhat intermediate with not enough marked differences to separate it from the other groups.

*Colony characters.* The numerous platings of the cultures and reisolations from nodules gave abundant opportunity for the study of both surface and deep colonies. Plates were always made with yeast-water mannitol agar.

Colony development on any of the media was slow. Colonies made their appearance only after ten days or two weeks, and did not reach their maximum size for three weeks. Plate cultures grown at 28°C. and containing not more than 25 or 30 colonies were the most characteristic. Careful study of all the strains revealed two types of colonies.

*Type A.* The surface colony was circular, never more than 3 mm. in diameter, convex, smooth, vitreous, quite dense, white and glistening. The edge is entire, smooth, and sharply defined. The internal structure is usually amorphous to very finely granular. The deep colony is very dense, usually boat- or lens-shaped with very sharp angles at the points. On account of the density the internal structure is amorphous. Both the preceding forms of this type of colony are shown in plate 1, figure 2.

*Type B.* In this type the colonies on the surface were usually circular, sometimes five or six millimeters in diameter, flat or raised, smooth, transparent to translucent, seldom conspicuously white and often dull. The edge varied from entire to undulate. The internal structure showed the greatest variation from type A. The flat thin growth corresponded to that of the streak cultures and the nearly transparent film was quite open in structure.

The colonies under the surface of the agar varied from elliptical to nearly spherical. Very few of these showed the sharply pointed and angular forms exhibited by type A. Their internal structure tended to be open and showed a mottled appearance unlike type A. The differences in the surface and deep colonies of the two types can be compared in plate 1. The small white points in the culture medium were particles of calcium carbonate.

### *Morphology of the strains*

Careful morphological studies were made of the organisms of each strain. Stained and hanging drop preparations were examined from each of the culture media where growth took place. The size and appearance of the cells in the cultures were found to vary with the composition and reaction of the medium, as well as with the kind and concentration of the staining solution used. Cells stained with weak dyes such as methylene blue, and dilute solutions of strong dyes, such as Ziehl's carbol fuchsin, were often granular or vacular in appearance. Such cells were confusing as to their actual size, and did not make good objects for measurements.

*Size and shape of the cells.* The preparations were made from 48-hour-old yeast-water mannitol agar streak cultures, which had been grown at 28°C. The air-dried and fixed organisms were stained one minute with regular Ziehl's carbol fuchsin. After thorough washing and drying the measurements were made with a 1.8-mm. oil immersion objective and filar micrometer.

Comparison of the measurements showed very little difference in the size and morphology of the strains. Strains 5 and 6, which are culturally quite different from the others, appeared on the average to be shorter in length but were about the same width. The difference, however, is not enough to be significant.

*Motility of cells of strains.* Since the first isolation of the nodule bacteria, most observers have agreed that they are motile organisms and possess flagella. However, as to the number and arrangement of the flagella, there has been considerable disagreement. The recorded observations of Beijerinck (2), Greig-Smith (21), De Rossi (14), Kellerman (28), Zipfel (43), Prucha (34), Wilson (42), Barthel (1), Fred and Davenport (18), Burrill and Hansen (6),

and Shunk (35) indicate that there are at least two types of flagellation of the legume bacteria. Although all strains of the organisms in six of the seven common cross-inoculating legume groups (clover, alfalfa, peas, soybeans, cowpeas, hog bean, lupine) have not been stained, the data that have been reported seem to justify the later conclusion of Hansen (23), that the legume bacteria are of two types, viz: peritrichous and monotrichous.

As practically all successful observers, except Wilson (42), have found one flagellum on the organism of *Soja max*, it was deemed advisable to stain the flagella of the eight strains being studied. The fact that there was considerable difference in cultural characters between some of the strains intimated that there might be a difference in the number and distribution of their flagella. That such was not the case was discovered later.

### *Method of staining flagella*

Some difficulty was experienced at first in securing a medium in which motile organisms would develop. During the course of some other work the solution evolved by Buchanan (4) was used. In this water-clear medium, all strains produced a visible clouding in 48 hours at 28°C., and after four or five days, a heavy, very finely divided turbidity. Microscopic examination of each of the cultures, after 48 hours incubation, showed all of them to be actively motile.

Attempts to stain flagella from these cultures were unsuccessful because the films would not fix. Transfers were made from 24-hour Buchanan's solution cultures to yeast-water mannitol agar slants. These cultures were grown 48 hours at 28°C., when there was enough growth for the preparation of films.

Spreads were made quickly on very thin cover glasses and were air dried. The mordant used was essentially that of Burrill and Hansen (6), except that the hydrogen-ion concentration was altered.

The flagella of each of the eight strains were stained. In every case the flagella were single (monotrichous) and attached at a corner (plate 2, figs. 1, 2 and 3). As the preparations of all the cultures were very much alike, photomicrographs of only three are shown. These are strains 1, 4 and 6 respectively.

*Tendency towards Gram's stain.* The relation of bacteria to the basic anilin dyes has received much consideration in recent years. The differentation of bacteria by means of the Gram method of staining is considered important from the standpoint of their susceptibility to basic anilin dyes like crystal violet (hexamethyl pararosanilin chloride). The recent experiments of Burke (5), Hucker (25), and Hucker and Conn (26) seem to show that the relation of any bacterium to Gram's stain is quite variable and dependent upon many conditions.

In order to establish, as well as possible, the tendency of the eight strains to the Gram's stain; 24-hour, 48-hour and 5-day-old cultures were stained by the Burke (5) technique and with the technique recommended by Hucker and Conn (26). The results were negative with both methods. There was some varia-

tion, but in no case was there enough stain retained to warrant calling any of the cultures Gram positive.

## PHYSIOLOGY OF STRAINS

### *Gum production*

The slimy nature of cultures of the legume bacteria has been observed by all working with these organisms. The composition and conditions governing the production of gum have been carefully investigated by Buchanan (4). The mechanism of production and composition of the gum has also been studied by Greig-Smith (22), and Fred (17).

In order to determine whether the quantity of gum produced might account for some of the differences in cultural characters, a series of solution cultures was made. As other investigators had shown a solution rich in nitrogen and carbohydrates to promote gum production, Buchanan's (4) solution was used. This solution was prepared in large quantities as needed. For the determination of gum production, 500-cc. portions of this solution adjusted to pH 6.8, were sterilized in 1000-cc. flasks. After cooling, each of the flasks was inoculated with 1 cc. of a fresh culture of the respective strains. The flasks were sealed with tin foil and incubated 6 weeks at 28°C. The cultures began to show a slight turbidity in 3 days. In 10 days there was a dense, uniformly fine turbidity. At the end of 3 weeks there was a heavy precipitate in addition to the turbidity. The appearance of the flasks was much the same after 6 weeks.

In preliminary trials the 95 per cent alcohol used by Buchanan (4) was tried. The results were only partly satisfactory. With some strains the precipitation was good; with others—5 and 6—there was practically no precipitation. Among other precipitating agents, acetone used by Fred (17) was found to be the most satisfactory. Most of the cultures precipitated in a short time, while others required 24 hours or more in the cold. One and one-half volumes of acetone was found to give the most satisfactory precipitation.

After precipitation, the gum was washed in several changes of distilled water and reprecipitated in tall glass cylinders. The volume of the solution was reduced to approximately 100 cc. by evaporation before filtration. The gum from the various strains was filtered through weighed Gooch filters, washed and dried. The gain in weight of the filter was taken as the gum in the culture. The gum production of the eight strains of soybean bacteria is given in table 2 as gum per 100 cc. of medium. The results show that there was great variation in gum production, and that the quantity of gum produced is comparable with the cultural characters of the strains. Strain 3 produced the greatest amount of gum, and belongs in the group producing the most abundant growth on solid media. Similarly strains 5 and 6 produced the smallest quantity of gum and produced the thinnest growth on solid media.

The quantities of gum found were somewhat lower than those reported by

Fred (17), and much lower than the results reported by Greig-Smith (22). The soybean organisms are of the monotrichous type, and according to Burrill and Hansen (6) do not produce as much slime as the peritrichously flagellated types.

*Relation of strains to reaction of medium*

The importance of true acidity—hydrogen-ion concentration—in biological processes has been well established by the classical researches of Sorensen (38) Clark and Lubs (11, 12), Dernby (15), Michaelis (32) and a host of other workers. The early contention of Maze (31) that there are acid-resistant and acid-sensitive types of the legume bacteria, which may become accustomed to a changed reaction, was not confirmed by Burrill and Hansen (6). These authors state:

> The reaction of artificial media does not break or change the special adaptations, nor is the organism modified at all so far as its power to produce nodules is concerned.

TABLE 2

*Gum production by different strains of soybean bacteria*

Buchanan's solution. 6 weeks at 28°C.

| STRAIN | DRY GUM PER 100 CC. OF MEDIUM |
|---|---|
| | *mgm.* |
| 1 | 135.00 |
| 2 | 104.00 |
| 3 | 144.00 |
| 4 | 109.00 |
| 5 | 42.00 |
| 6 | 52.00 |
| 7 | 107.00 |
| 8 | 67.00 |

However, they did not investigate the relation of their strains to the true acidity of the culture media, or to the reaction of the sap of the host plants.

In an extensive study of the influence of reaction on the legume bacteria, Fred and Davenport (18), found the critical and optimal hydrogen-ion concentrations of twenty-one different strains to vary over quite a wide range of the pH scale. A striking fact in their experiments was the sharp line of demarcation between a reaction permitting growth and one entirely preventing it. With the exception of the nodule bacteria of *Medicago sativa*, of which there were seven strains, and four strains of the organisms of *Lupinus;* they investigated few homologous strains of other host plants. These homologous strains when grown in mannitol solution, at varying hydrogen-ion concentrations, all showed the same critical pH values. This would indicate that all strains of legume bacteria, of the same species of host plants, have protoplasm which is affected by the same reaction.

The data reported here are based upon three series of parallel tests. Previ-

ous experience had shown that Buchanan's solution, as a liquid medium for the soybean nodule bacteria, was superior to any other available. Its colorless, water-clear appearance makes the colorimetric determination of reaction easy and accurate. The heavy turbidity which develops when the bacteria grow in the solution is also easily detected.

The culture solution was prepared in large quantities and distributed in 500-cc. flasks for use. The reaction of the solution was carefully adjusted by means of electrometrically checked buffer solutions prepared according to Clark (10). The final check of the sterilized solutions was made electrometrically. In no case were solutions used where there was a discrepancy of 0.04 pH. The adjust-

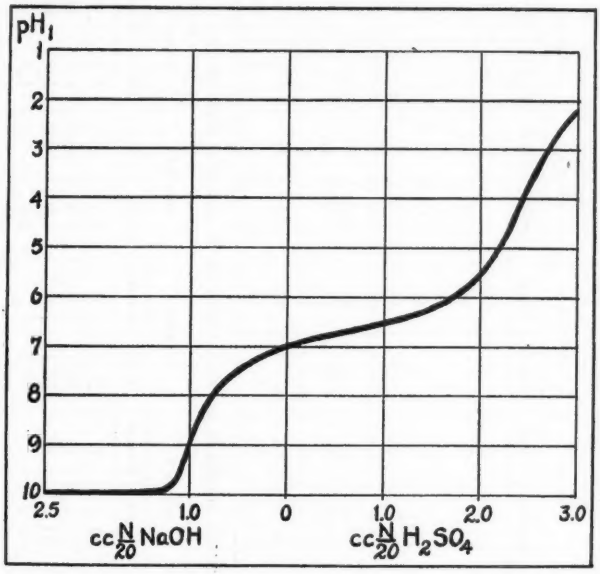


FIG. 1. TITRATION CURVE SHOWING BUFFER EFFECT OF BUCHANAN'S SOLUTION

ment of the reaction of the different series of solutions was made on the acid side with 0.05 *N* sulfuric acid, and on the alkaline side with 0.05 *N* sodium hydroxide.

Titrations of the separate constituents of Buchanan's solution showed that the buffer effect is due almost entirely to the acid potassium phosphate. A very slight buffer effect is produced by the peptone. The titration curve of the medium is shown in figure 1, which indicates the strongest buffer action of the solution to be between pH 7.5 and 6.0.

Distilled water suspensions of organisms were used for the inocula. These were prepared by washing into sterile flasks the organisms from several yeast-water mannitol agar slants. The flasks were vigorously shaken to make the

TABLE 3

*Culture tests*

Growth of eight strains of soybean nodule bacteria in Buchanan's solution of varied reaction. 2 weeks at 28°C.

| pH | STRAIN 1 | | STRAIN 2 | | STRAIN 3 | | STRAIN 4 | | STRAIN 5 | | STRAIN 6 | | STRAIN 7 | | STRAIN 8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Growth | Streak | Growth | Streak | Growth | Streak | Growth | Streak | Growth | Streak | Growth | Streak | Growth | Streak | Growth | Streak |
| 4.0 | − | O. | − | O. | − | O. | − | O. | − | T. | − | T. | − | O. | − | O. |
| 4.2 | − | O. | − | O. | − | O. | − | O. | ++ | G. | ++ | G. | − | O. | − | O. |
| 4.4 | − | O. | − | O. | − | O. | + | G. | ++ | G. | ++ | G. | − | O. | + | T. |
| 4.6 | + | G. | + | G. | + | G. | + | G. | ++ | G. | ++ | G. | + | T. | ++ | G. |
| 4.8 | + | G. | + | G. | ++ | G. | ++ | G. | +++ | G. | +++ | G. | + | G. | ++ | G. |
| 5.0 | + | G. | ++ | G. | ++ | G. | +++ | G. | +++ | G. | +++ | G. | ++ | G. | ++ | G. |
| 5.2 | ++ | G. | ++ | G. | ++ | G. | +++ | G. | +++ | G. | +++ | G. | ++ | G. | +++ | G. |
| 5.4 | ++ | G. | ++ | G. | +++ | G. | +++ | G. | +++ | G. | +++ | G. | ++ | G. | +++ | G. |
| 5.6 | +++ | G. | +++ | G. | +++ | G. | +++ | G. | ++ | G. | ++ | G. | +++ | G. | +++ | G. |
| 5.8 | +++ | G. | +++ | G. | +++ | G. | +++ | G. | ++ | G. | ++ | G. | +++ | G. | +++ | G. |
| 6.0 | +++ | G. | +++ | G. | +++ | G. | ++ | G. | ++ | G. | ++ | G. | +++ | G. | ++ | G. |

Bacteria in millions per cubic centimeter as shown by plate counts

| | STRAIN 1 | | STRAIN 2 | | STRAIN 3 | | STRAIN 4 | | STRAIN 5 | | STRAIN 6 | | STRAIN 7 | | STRAIN 8 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks | Initial | 2 weeks |
| 4.0 | 10.2 | 0 | 14.2 | 0 | 11.2 | 0 | 25.2 | 0 | 5.9 | 0 | 4.8 | 0 | 22.0 | 0 | 9.4 | 0 |
| 4.2 | 10.2 | 0 | 14.2 | 0 | 11.2 | 0 | 25.2 | 0 | 5.9 | 29.9 | 4.8 | 32.0 | 22.0 | 0 | 9.4 | 0 |
| 4.4 | 10.2 | 0 | 14.2 | 0 | 11.2 | 0 | 25.2 | 64.8 | 5.9 | 50.0 | 4.8 | 48.5 | 22.0 | 0 | 9.4 | 0 |
| 4.6 | 10.2 | | 14.2 | | 11.2 | | 25.2 | | 5.9 | | 4.8 | | 22.0 | | 9.4 | |
| 4.8 | 10.2 | | 14.2 | | 11.2 | | 25.2 | | 5.9 | | 4.8 | | 22.0 | | 9.4 | |
| 5.0 | 10.2 | 32.0 | 14.2 | 22.4 | 11.2 | 28.0 | 25.2 | 355.0 | 5.9 | 90.0 | 4.8 | 112.0 | 22.0 | 50.0 | 9.4 | 420.0 |
| 5.2 | 10.2 | | 14.2 | | 11.2 | | 25.2 | | 5.9 | | 4.8 | | 22.0 | | 9.4 | |

| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5.4 | 10.2 | | 14.2 | | 11.2 | | 25.2 | | 5.9 | | 4.8 | | 22.0 | | 9.4 | |
| 5.6 | 10.2 | 347.0 | 14.2 | 250.0 | 11.2 | 420.0 | 25.2 | 487.0 | 5.9 | 86.0 | 4.8 | 100.0 | 22.0 | 309.0 | 9.4 | 500.0 |
| 5.8 | 10.2 | | 14.2 | | 11.2 | | 25.2 | | 5.9 | | 4.8 | | 22.0 | | 9.4 | |
| 6.0 | 10.2 | 625.0 | 14.2 | 420.0 | 11.2 | 443.0 | 25.2 | 382.0 | 5.9 | 25.6 | 4.8 | 49.2 | 22.0 | 250.0 | 9.4 | 311.0 |

+, indicates slight growth.
++, indicates medium growth.
+++, indicates greatest growth.
G., indicates good typical growth.
T., indicates trace of growth.
O., indicates no growth.

suspensions as uniform as possible. Plate counts of the suspensions, after dilution for use as inocula, showed a range of 5 to 25 millions per cubic centimeter.

The sets of tubes at the different pH values were all inoculated at the same time. Each tube received 1 cc. of inoculum. Plate counts of the suspensions used for inocula were made at the time of inoculation. All cultures were grown at 28°C. for 2 weeks. Observations were made after 1 and 2 weeks. The preliminary experiment was set up with solutions increasing by increments of 0.5 pH, from pH 2.5 to pH 9.0. All other conditions were as stated above, except that no plate counts were made. All of the tubes, which were in duplicate in each set, were streaked on yeast-water mannitol agar at the end of 2 weeks. The results of this experiment showed that the limiting and optimum hydrogen-ion concentrations of Buchanan's solution were not the same for all the strains; strains 5 and 6 grew at a lower pH value than the other strains. As judged by turbidity their optimum also was lower.

In order to fix more closely the limiting hydrogen-ion concentrations for the strains, a second experiment was set up. In this experiment the same procedures were followed as before, except that the solutions were prepared to differ by increments of 0.2 pH and plate counts were made. The plate counts were made of the diluted inocula, and of part of the cultures after a period of 2 weeks. The growth as shown by turbidity, and the plate counts in millions per cubic centimeter are given in table 3.

Examination of the table shows that the limiting range of pH for the eight strains is not the same for all. Further the agar-streak transfers from the cultures in Buchanan's solution show very few organisms to have survived where the solution remained clear.

These results tend to show that the lower limit of growth for strains 1, 2, 3 and 7 is some place between pH 4.5 and 5.0; while that for strains 5 and 6 is between pH 4.5 and 4.0. Strains 7 and 8 seem to hold an intermediate position. The optima for strains 5 and 6 also seem to be lower than for the other strains. It is interesting to note that the optimum reaction for growth of all strains is below pH 7.0 and nearer 6.0 than 7.0. In fact, all strains in this medium grow poorly above pH 7.0 and do not grow above pH 8.0. The limiting action of changes on the alkaline side of the neutral point was not nearly so sharp and well defined as on the acid side. The differences that were apparent in the preliminary experiment are more evident in the second trial. Strains 1, 2, 3, and 7 tend to grow best nearer neutrality, while strains 5 and 6 seem to grow best at more acid hydrogen-ion concentrations. Strains 4 and 8 seem to have an intermediate position when compared with the other strains. These comparisons based upon the turbidity produced in Buchanan's solution check very well with the plate counts. Comparisons of the plate counts show the greatest reproduction of strains 1, 2, 3 and 7 to have taken place between pH 5.6 and 6.0. The greatest reproduction of strains 5 and 6 took place below pH 5.6. The counts also show that the latter strains grow at

hydrogen-ion concentrations as low as pH 4.2. No other strains grew in solutions so acid. The counts of cultures 4 and 8 tend to confirm the conclusions from the appearance of the cultures. These strains gave the greatest growth at a hydrogen-ion concentration of about pH 5.6. These strains appeared to be intermediate between the groups 1, 2, 3, 7 and 5, 6.

The critical hydrogen-ion concentrations of the eight strains were quite different. A classification of them based upon their growth in Buchanan's solution is as follows:

| | STRAINS | CRITICAL pH | OPTIMUM pH |
|---|---|---|---|
| Acid resistant | 5, 6 | 4.1 | 4.8 to 5.5 |
| Acid sensitive | 1, 2, 3, 7 | 4.5 | Above 5.8 |
| Intermediate | 4, 8 | 4.3 | Above 5.4 |

The surprising effects of the critical hydrogen-ion concentrations are shown well by the photographs of strains 2, 4, and 5 in plates 3 and 5. Where the organisms grew, the variations in turbidity, perceptible only by transmitted light are not apparent in the photographs.

The differences in the relation of the strains to the reaction of the nutrient soution, harmonize with the differences noted in cultural characters. Such characteristics indicate that although the strains are morphologically alike they are physiologically different.

The final hydrogen-ion concentration and titratable acidity were determined in the belief that they might show some differences between the strains. The determinations show for all the strains practically no increase in true acidity (lowering of the pH) and in practically every case an actual reduction of the total acidity. The failure of the legume bacteria to change the true acidity of culture solutions has been observed by Fred and Davenport (18). They have suggested that the small amount of sugar consumed by the organisms accounts for this peculiarity.

The decrease in the titratable acidity may be due either to the production of ammonia or to the utilization of acid radicals in the solution. It has been noted above that all strains tend to make milk strongly alkaline after 2 weeks time. Plate cultures containing casein, and analysis of milk cultures by the Van Slyke method failed to disclose any changes in the milk proteins.

### *Susceptibility of the strains to dyes*

The differentiation of closely related strains of bacteria by means of anilin dyes, such as gentian violet, has been extensively studied by Churchman (8) and Churchman and Michael (9). The most important constituent of gentian violet which affects Gram-positive bacteria has been shown by Crossley (13) to be hexamethyl pararosanilin chloride, or crystal violet. The following experiments were made with pure recrystallized crystal violet.

The earlier studies of the strains showed that they were all Gram negative. This property would place them in the dye-resistant group. However, the findings of Churchman (8), Churchman and Michael (9), and Skinner and Murray (37) have shown that strains closely related may be differentiated by means of dyes.

More recently Vandecaveye (40) has reported some preliminary results with dyes in the study of the legume bacteria. His results show concentrations of crystal violet as great as 1:2000 to give differential results. In the report the strains of nodule bacteria and culture media used are not given.

Buchanan's solution was used for the tests. A standard solution of the dye was prepared by dissolving 0.1 gm. of the crystalline salt in 100 cc. of distilled water. Different amounts of this solution were diluted with nutrient solution in preparing the dilutions for use. In a preliminary experiment it was dis-

TABLE 4

*Effect of crystal violet on the growth of soybean nodule bacteria*

Buchanan's solution. 2 weeks at 28°C.

| STRAIN | CONTROLS | DILUTION OF CRYSTAL VIOLET | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 2,500 | 5,000 | 7,500 | 10,000 | 20,000 | 50.000 | 100,000 | 200,000 |
| 1 | +++ | − | − | − | − | − | − | + | ++ |
| 2 | +++ | − | − | − | − | − | − | + | ++ |
| 3 | +++ | − | − | − | − | − | − | + | ++ |
| 4 | +++ | − | − | − | − | − | + | + | ++ |
| 5 | +++ | − | − | − | + | + | ++ | ++ | +++ |
| 6 | +++ | − | − | − | + | ++ | ++ | ++ | +++ |
| 7 | +++ | − | − | − | − | − | − | + | + |
| 8 | +++ | − | − | − | − | − | + | ++ | ++ |

+ indicates slight growth.
++ indicates medium growth.
+++ indicates greatest growth.

covered that some strains grew in a 1:10,000 dilution of crystal violet while others grew in a concentration of 1:100,000. A second series of cultures was prepared with dilutions of dye covering a wider range. In this series of cultures 9 cc. of the culture solution, containing the dye, was inoculated with 1 cc. of a suspension of the organisms. The cultures were incubated at 28°C. for 2 weeks. At the end of the incubation period, transfers were made from the dye cultures and from cultures without dye, to yeast-water mannitol agar slants. After 2 weeks at 28°C. these slant cultures were examined for growth. The results of the tests are given in table 4. Inspection of the table shows that all the strains are dye-resistant, some much more so than others. For example, strains 5 and 6 growing in a concentration of 1:10,000 can endure ten times the amount tolerated by strains 1, 2, 3 and 7 growing in 1:100,000. Strains 4 and 8 appear to come into an intermediate position in relation to the other two groups.

Among other dyes tried, were acid fuchsin and malachite green. Both of these dyes failed to give the differentiation of the strains shown by crystal violet. Malachite green had more of an inhibiting effect on strains 1, 2, 3, and 7 than on strains 5 and 6. All strains grew in dilutions as low as 1:5000, and the differentiation was not distinct.

The differences in the strains brought out by crystal violet support the differences shown by the strains in cultural characters, in gum production, and in relation to hydrogen-ion concentration.

### *Serological relations of strains*

In the investigation of pathogenic microorganisms serological methods have come to play an important part. The segregation, by means of serological tests, of otherwise identical strains of bacteria has become of great practical importance.

It has been shown by Butterfield and Neill (7) that, regardless of geographic distribution, practically all agglutinable strains of meningococci may be classified under four types. Such a separation would be impossible by means of the usual microscopic, cultural and inoculation tests. The correlation of sixteen strains of *Bacillus bovisepticus* serologically and culturally in three distinct groups has been demonstrated by Jones (27).

As an inconsistancy in correlation we have the results of Evans (16) with forty-nine strains of *Brucella melitensis*. The species, based upon the cultures used, was differentiated into seven serological groups. One of the most important of these included human strains of the Malta Fever organism, and several strains of *abortus of bovine* origin. The agglutination test does not differentiate between these organisms, while at the same time one possesses a salient pathogenesis for man and the other does not. Such strains, though alike culturally and serologically, and unlike pathogenically, can hardly be considered identical.

Although plant inoculation has been and is at present the criterion for identification of the nodule bacteria, considerable serological data have been secured concerning them. The first serological studies of the nodule bacteria were made by Zipfel (43). By means of a limited number of cultures, isolated directly from nodules, he showed a serological relation between the nodule bacteria of *Pisum sativum* and *Phaseolus vulgaris*.

In a more critical study of the nodule bacteria of seven families of *Leguminosae;* Simon (36), compared the host plant and the serological relations of pure cultures of the bacteria. His results showed five cross-inoculating and cross-agglutinating groups.

The agglutination, complement fixation and precipitation reactions of eighteen different strains of legume bacteria were studied by Klimmer and Krüger (29). These investigators found the serological properties of the strains quite characteristic, definite, and correlated with cross-inoculation of host plants. As a result of the very careful serological study of many strains of

nodule bacteria, Vogel and Zipfel (41), came to the conclusion that the nodule bacteria are not all one species. These authors conclude that the serological properties of the bacteria are definite, inheritable characteristics, but that the plant-infecting power and the nitrogen-fixing ability are direct life characteristics. They report no nitrogen fixation studies.

Stevens (39) has made a careful serological study of fifty-five strains of the legume bacteria. The cultures represented the seven groups of cultivated legumes of this country. His results confirm the work of other investigators with respect to host plant groups and serological properties of the bacteria. He was further able to show that the strains for the same host plants could be divided into distinct serological groups.

More recently Bialosuknia and Klott (3) have reported serological tests with several strains of legume nodule bacteria. With carefully pot-tested cultures they found the serological properties of the strains to be constant. These authors made the interesting observation that more than one serological strain may form nodules on the same plant, but only one serological type is found in the same nodule.

### *Immunological tests*

The cultures used in this and the preceding studies were from the same sources as those used by Stevens (39). The cultures had been in use for a number of years. They had been reisolated from plants several times, when the agglutination tests were made. All except two of the eight strains had been used for greenhouse and field experiments. These two, strains 7 and 8, were acquired after much work had been done with the other strains. During the progress of the agglutination work all stock and reisolated strains were grown on the regular yeast-water mannitol agar.

Rabbits were used for the production of the immune sera. The animals were albinos, of one litter, and weighed approximately three kilograms each, when they were immunized. The same animal was always used for each strain of bacteria.

The antigens were prepared from 5-day, agar-slant cultures, grown at 28°C. The growth in each tube was washed off with 0.85 per cent sodium chloride solution and transferred to a sterile 150-cc. Erlenmeyer flask. Duplicate cultures were washed off and added to those in the flask until suspensions, somewhat heavier than needed for use, were obtained. The suspensions were then standardized by the method described by Gates (20). Inocula for animal injection were prepared in a similar manner.

The animals were injected intraperitoneally with the suspensions of the organisms in salt solution. Each animal was given three injections as follows: At the start, 1 cc.; after 4 days, 2.5 cc.; 8 days from start, 5 cc.

Hourly temperatures taken after each injection showed a maximum rise after the second injection, of about three degrees. There were very slight rises after the other injections.

The animals were bled directly from the heart. They were fastened to an animal board while 10 cc. of blood was drawn under strictly aseptic conditions. The blood was drawn with a sterile glass-barreled syringe equipped with a number 19 slip-on needle.

The warm blood was ejected into sterile test tubes, and placed in the ice box to clot. After 24 hours the clots were carefully separated from the sides

TABLE 5

*Serum of Strain 2*

| ANTIGENS | | DILUTIONS OF SERUM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Source | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| 1 | O. | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | P. | − | − |
| | R. | ++ | ++ | ++ | ++ | ++ | + | + | + | − | − | − |
| 2 | O. | ++ | ++ | ++ | ++ | ++ | ++ | + | P. | − | − | − |
| | R. | ++ | ++ | + | + | + | + | − | − | − | − | − |
| 3 | O. | ++ | ++ | ++ | ++ | ++ | ++ | + | P. | − | − | − |
| | R. | ++ | ++ | ++ | ++ | + | + | + | P. | − | − | − |
| 4 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | + | P. | − | − | − | − | − | − | − | − | − |
| 5 | O. | + | − | − | − | − | − | − | − | − | − | − |
| | R. | P. | + | − | − | − | − | − | − | − | − | − |
| 6 | O. | − | − | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |
| 7 | O. | ++ | ++ | + | + | + | + | − | − | − | − | − |
| | R. | ++ | + | + | + | + | + | − | − | − | − | − |
| 8 | O. | + | − | − | − | − | − | − | − | − | − | − |
| | R. | + | − | − | − | − | − | − | − | − | − | − |

++, clearly defined precipitation with clump at bottom of tube and no floating particles.
+, precipitation consisting of clumps some at bottom, some floating.
P., slight tendency to clumping but no separation.
−, no change.

of the tubes by means of sterile glass rods. The coagula soon contracted and expressed the clear serum.

In making the tests the following dilutions of serum were used: 1, 1:20; 2, 1:50; 3, 1:100; 4, 1:250; 5, 1:500; 6, 1:1000; 7, 1:2000; 8, 1:5000; 9, 1:7500; 10, 1:10,000 and 11, 1:20,000. The antigen, prepare as described earlier, was first put into test tubes 9 by 75 mm. In each tube 1 cc. was used. To each of

these tubes, numbered as above, was added the corresponding dilution of serum.

After thorough mixing the tubes were held 48 hours at 37°C. Control tubes were run with each set of tests.

In tables 5, 6, 7, and 8 are given the agglutination reactions of four of the representative strains with all the antigens. The results of the tests are given

TABLE 6

*Serum of strain 4*

| ANTIGENS | | DILUTIONS OF SERUM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Source | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| 1 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | − | − | − | − | − | − | − | − | − | − | − |
| 2 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | − | − | − | − | − | − | − | − | − | − | |
| 3 | O. | − | − | − | − | − | − | − | − | − | − | − |
| | R. | − | − | − | − | − | − | − | − | − | − | − |
| 4 | O. | ++ | ++ | ++ | ++ | ++ | ++ | + | P. | − | − | − |
| | R. | ++ | ++ | ++ | + | + | + | + | P. | − | − | − |
| 5 | O. | P. | − | − | − | − | − | − | − | − | − | − |
| | R. | − | − | − | − | − | − | − | − | − | − | − |
| 6 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | |
| 7 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | − | − | − | − | − | − | − | − | − | − | − |
| 8 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |

++, clearly defined precipitation with clump at bottom of tube and no floating particles.
+, precipitation consisting of clumps some at bottom, some floating.
P., slight tendency to clumping but no separation.
−, no change.

for the original stock cultures 5 years old, marked O.; and for the reisolated cultures of the same strains, marked, R. Comparisons of the data show the strains to be serologically unlike. The differences are consistant; the old stock cultures giving the same agglutinations as the newly isolated ones. Classification of the homologous strains reveals four serological groups shown in table 9, as follows:

Group I, strains 1, 2, 3 and 7
Group II, strain 4
Group III, strain 8
Group IV, strains 5 and 6

It is interesting to note the similarity of the serological groupings and the other physiological characteristics which have been described. The analogy

TABLE 7

*Serum of strain 5*

| ANTIGENS | | DILUTIONS OF SERUM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Source | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| 1 | O. | P. | − | − | − | − | − | − | − | − | − | − |
| | R. | + | − | − | − | − | − | − | − | − | − | − |
| 2 | O. | P. | − | − | − | − | − | − | − | − | − | − |
| | R. | + | + | P. | − | − | − | − | − | − | − | − |
| 3 | O. | P. | − | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |
| 4 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | + | P. | − | − | − | − | − | − | − | − | − |
| 5 | O. | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | P. |
| | R. | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | + |
| 6 | O. | + | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + | P. |
| | R. | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | ++ | + | + |
| 7 | O. | − | − | − | − | − | − | − | − | − | − | − |
| | R. | + | − | − | − | − | − | − | − | − | − | − |
| 8 | O. | P. | − | − | − | − | − | − | − | − | − | − |
| | R. | + | − | P. | − | − | − | − | − | − | − | − |

++, clearly defined precipitation with clump at bottom of tube and no floating particles.
+, precipitation consisting of clumps some at bottom, some floating.
P., slight tendency to clumping but no separation.
−, no change.

is especially evident when group I and group IV are compared. In cultural characters, gum production, sensitivity to dyes, and critical hydrogen-ion concentrations they are quite different. Groups II and III, each consisting of a single strain, appear to have an intermediate position. These intervening groups, although clearly isolated serologically, are not so variant in other respects. Several of their attributes, as cultural characters, seem to place them closer to group I than group II.

TABLE 8

*Serum of strain 8*

| ANTIGENS | | DILUTIONS OF SERUM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Strain | Source | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| 1 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | + | − | − | − | − | − | − | − | − | − | − |
| 2 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |
| 3 | O. | + | − | − | − | − | − | − | − | − | − | − |
| | R. | + | P. | − | − | − | − | − | − | − | − | − |
| 4 | O. | + | P. | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |
| 5 | O. | − | − | − | − | − | − | − | − | − | − | − |
| | R. | + | P. | − | − | − | − | − | − | − | − | − |
| 6 | O. | − | − | − | − | − | − | − | − | − | − | − |
| | R. | P. | − | − | − | − | − | − | − | − | − | − |
| 7 | O. | + | − | − | − | − | − | − | − | − | − | − |
| | R. | + | − | − | − | − | − | − | − | − | − | − |
| 8 | O. | ++ | ++ | ++ | ++ | ++ | + | + | − | − | − | − |
| | R. | ++ | ++ | ++ | ++ | ++ | ++ | + | + | − | − | − |

++, clearly defined precipitation with clump at bottom of tube and no floating particles.
+, precipitation consisting of clumps some at bottom, some floating.
P., slight tendency to clumping but no separation.
−, no change.

TABLE 9

*Serological groups of eight strains of soybean nodule bacteria*

| GROUPS | ANTIGENS | MAXIMUM TITER OF ANTISERA (STRAINS) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 7 | 4 | 8 | 5 | 6 |
| I | 1 | 1:5,000 | 1:2,000 | 1:2,000 | 1:1,000 | − | − | − | − |
| | 2 | 1:5,000 | 1:5,000 | 1:5,000 | 1:1,000 | − | − | − | − |
| | 3 | 1:2,000 | 1:2,000 | 1:5,000 | 1:2,000 | − | − | − | − |
| | 7 | 1:2,000 | 1:1,000 | 1:1,000 | 1:2,000 | − | − | − | − |
| II | 4 | − | − | − | − | 1:2,000 | − | − | − |
| III | 8 | − | − | − | − | − | 1:5,000 | − | − |
| IV | 5 | − | − | − | − | − | − | 1:20,000 | 1:20,000 |
| | 6 | − | − | − | − | − | − | 1:20,000 | 1:20,000 |

Inasmuch as *B. radiobacter* is often confused with the legume nodule bacteria; its serological properties were compared severally with the eight strains of soybean bacteria. The data of table 10 show the highly specific nature of the radiobacter antiserum, and also furnish serological proof that none of the cultures were contaminated with this organism.

TABLE 10

*B. radiobacter serum and antigen reactions*

| ANTIGENS | DILUTIONS OF RADIOBACTOR SERUM | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| *B. radiobacter* | ++ | ++ | ++ | ++ | ++ | + | + | + | P. | − | − |
| 1 | − | − | − | − | − | − | − | − | − | − | − |
| 2 | P. | − | − | − | − | − | − | − | − | − | − |
| 3 | P. | − | − | − | − | − | − | − | − | − | − |
| 4 | + | P. | − | − | − | − | − | − | − | − | − |
| 5 | − | − | − | − | − | − | − | − | − | − | − |
| 6 | − | − | − | − | − | − | − | − | − | − | − |
| 7 | P. | − | − | − | − | − | − | − | − | − | − |
| 8 | − | − | − | − | − | − | − | − | − | − | − |
| | DILUTIONS OF SOYBEAN SERA | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| | 1:20 | 1:50 | 1:100 | 1:250 | 1:500 | 1:1,000 | 1:2,000 | 1:5,000 | 1:7,500 | 1:10,000 | 1:20,000 |
| *B. radiobacter* | ++ | ++ | ++ | ++ | ++ | ++ | + | + | − | − | − |
| 1 | + | − | − | − | − | − | − | − | − | − | − |
| 2 | + | P. | − | − | − | − | − | − | − | − | − |
| 3 | + | − | − | − | − | − | − | − | − | − | − |
| 4 | + | − | − | − | − | − | − | − | − | − | − |
| 5 | − | − | − | − | − | − | − | − | − | − | − |
| 6 | − | − | − | − | − | − | − | − | − | − | − |
| 7 | − | P. | − | − | − | − | − | − | − | − | − |
| 8 | + | − | − | − | − | − | − | − | − | − | − |

++, clearly defined precipitation with clump at bottom of tube and no floating particles.
+, precipitation consisting of clumps some at bottom, some floating.
P., slight tendency to clumping but no separation.
−, no change.

## SUMMARY

Comparisons of all the data seem to indicate that there are two biotypes among the eight strains of *Pseudomonos radicicola* used in this investigation. Morphologically they are alike. Physiologically they are quite different.

The following descriptions apply in a general way, to the two types.

*Type A*

*Morphology:* Short, round ended rods 0.4 to 0.7$\mu$ by 2.0 to 3.0$\mu$ with one single flagellum attached at a corner of the cell, and occurring singly.

*Cultural characters:* On solid media, best growth on yeast-water mannitol agar. In liquid media, best growth in Buchanan's solution and milk.

*Yeast-water mannitol agar slant:* Moderate to abundant white, dense, glistening growth after 1 week at 28°C.

*Yeast-water mannitol agar colonies:* Colonies of this type are circular, dense, pearly white, opaque on the surface, and very dense, lens-shaped, sharply pointed or ellipsoidal when under the surface.

*Buchanan's solution:* Slight, uniform turbidity in 48 hours turning to a heavy, fine turbidity with somewhat viscous sediment in 2 weeks. No surface membrane, ring or pellicle.

*Litmus milk:* No apparent change at first. In two or three weeks strongly alkaline. No hydrolysis or coagulation of the casein. No serum zone formed. No reduction of the litmus.

*Meat infusion gelatin and broth:* No growth.

*Potato steak:* No growth.

*Gum production:* Above 100 mgm. per 100 cc. of Buchanan's solution.

*Reaction of Buchanan's solution:* Critical pH 4.5, optimum above 5.8.

*Serological properties:* Four of the eight cultures, studied serologically, produced homologous sera. Two other strains, although homologous serologically possess so many characteristics of type A that they may be considered slight modifications of the type. The type A strains rarely produced sera with a titer above 1:5000.

*Nodule production:* When type A is used to inoculate seeds or seedlings, the nodules are usually centrally located on the plant root system, and tend to become large and develop in clusters. This is true especially when the seeds are inoculated.

*Type B*

*Morphology:* Short, round ended rods 0.4 to 0.7$\mu$ by 2.0 to 3.0$\mu$ with one single flagellum attached at a corner of the cell, and occurring singly.

*Cultural characters:* On solid media, best growth on yeast-water mannitol agar. In liquid media, best growth in Buchanan's solution and milk.

*Yeast-water mannitol agar slant:* Scant to moderate, spreading, transparent to translucent, moist, growth after 1 week at 28°C.

*Yeast-water mannitol agar colonies:* Thin, spreading, circular transparent to translucent, circular surface colonies. Sometimes where they are small and more dense they take on a whitish appearance. The deep colonies are usually circular, to roundly oval, and contain open or transparent irregular areas.

*Buchanan's solution:* Slight, uniform turbidity in 48 hours, turning to a very fine, heavy turbidity with slight sediment in 2 weeks. No surface membrane, ring or pellicle.

*Litmus milk:* No apparent change at first. In 10 days to 3 weeks strongly alkaline. No hydrolysis or coagulation of the casein. No serum zone and no reduction of the litmus.

*Meat infusion gelatin:* No growth.

*Meat infusion broth:* After 10 days to 2 weeks a very fine, transparent turbidity appears which does not become heavy or precipitate. No ring or pellicle.

*Potato streak:* Very scant, moist, whitish growth after three or four weeks. Bacteroids are formed.

*Gum production:* Usually less than 100 mgm. per 100 cc. of Buchanan's solution.

*Reaction of Buchanan's solution:* Critical pH 4.1, optimum above 5.5.

*Serological properties:* This type produces sera of very high titer, 1:20,000 or higher; and does not agglutinate any strains of the other type.

*Nodule production:* The tendency of type B is to produce scattered nodules on the lateral roots of the plants. The nodules are usually smaller than those produced by type A, and usually do not form the large clusters around the main root so frequently as type A.

The types described may not be the only ones extant. In fact a more extensive study of a larger number of strains from a wider geographical distribution might reveal additional types. Such a study is contemplated.

Both types described produce nodules on three varieties of *Soja max* under carefully controlled greenhouse and under field conditions. The tendency of type A to form dense clusters of large nodules around the main root indicates a thorough early infection of the plant. Although type B produces some nodules around the main root, more of the nodules, which are usually not so large, are on the lateral roots. This type of nodule would appear to indicate a later infection of the plant.

The ability of one type to withstand greater acidity than the other, might enable such a strain of the organism to predominate in a very acid soil. In a composite culture used for seed inoculation the predominance of such a strain might lead to poor inoculation or to nodule production by an inferior strain. The different types may be influenced by variations in the hydrogen-ion concentration of the cell sap of the host plant; which is in turn influenced by the reaction and composition of the soil. Different combinations of these influences might produce the inconsistent field results sometimes observed.

## CONCLUSIONS

1. Not all strains of *Pseudomona radicicola* of *Soja max* are identical. The strains studied are identical morphologically but are not the same culturally or physiologically.

2. Two general types of the microorganisms, designated as type A and type B, have been studied and described.

3. In the strains studied the two types of the soybean nodule organism could be recognized culturally.

4. The serological reactions of the strains are very definite and quite constant, and do not lose or change this property after long transfer on artificial culture media, or after plant passage.

5. The serological properties of the nodule organism of *Soja max* cannot be used to identify any strain of the organism or the types. Two biotypes have been differentiated. The eight strains studied were composed of four serological groups. A larger collection of cultures from a wider geographical distribution might yield more groups.

6. The cultural and physiological properties of the strains of type A are not entirely correlated with their serological properties. Two distinct serological groups, except for limiting hydrogen-ion concentrations, and dye relations, belong in type A.

7. The critical hydrogen-ion concentrations of the strains are remarkably distinct and are characteristic of the types.

8. The inhibiting concentrations of the dyes are characteristic of the types. Crystal violet is superior for this purpose.

9. The ability to produce gum appears to be a type characteristic and may be related to the critical hydrogen-ion concentrations of the types.

10. The soybean nodule organism, or any of its types, cannot be recognized by any single laboratory test. The identity of species can be recognized by plant inoculation, the type only by determining all of its known properties.

11. The two groups, with their properties, do not represent different species; but distinct biotypes, each of which varies around a mean of its own.

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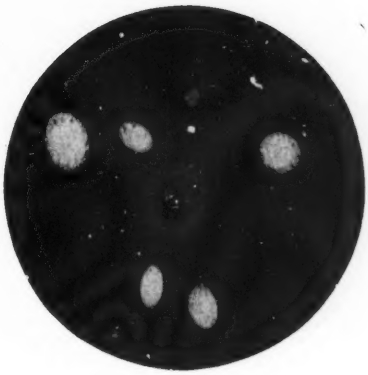
## PLATE 1

FIG. 1. Bacteriods developed by strain 5 on potato. Dilute carbolfuchsin. 1500×.

FIG. 2. Surface and deep colonies of type A after two weeks in yeast-water mannitol agar. 10×.

FIG. 3. Surface and deep colonies of type B after two weeks in yeast-water mannitol agar. 10×.



## PLATE 2

FIG. 1. Flagella preparation of the organisms of strain 1. 1500×.
FIG. 2. Flagella preparation of the organisms of strain 4. 1500×.
FIG. 3. Flagella preparation of the organisms of strain 6. 1500×.

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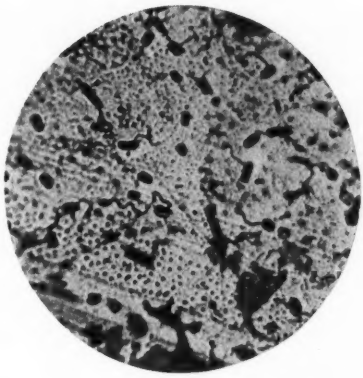

PLATE 3

CULTURES OF STRAIN 2 IN BUCHANAN'S SOLUTION AFTER 2 WEEKS AT 28°C.

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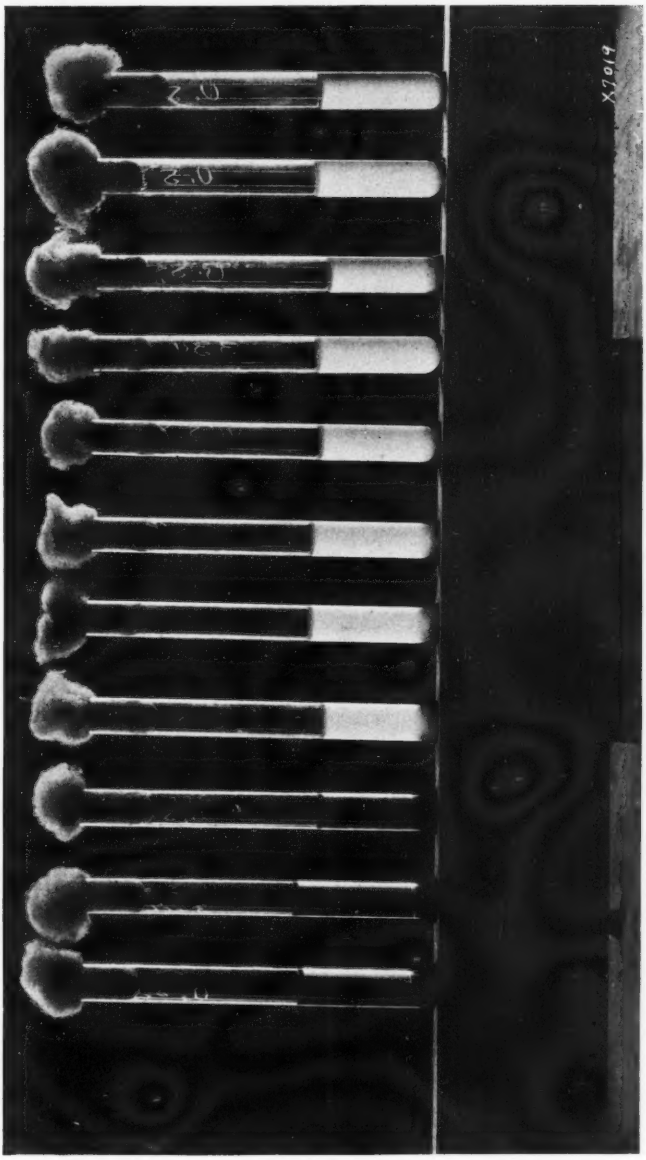

## PLATE 4

CULTURES OF STRAIN 4 IN BUCHANAN'S SOLUTION AFTER 2 WEEKS AT 28°C.

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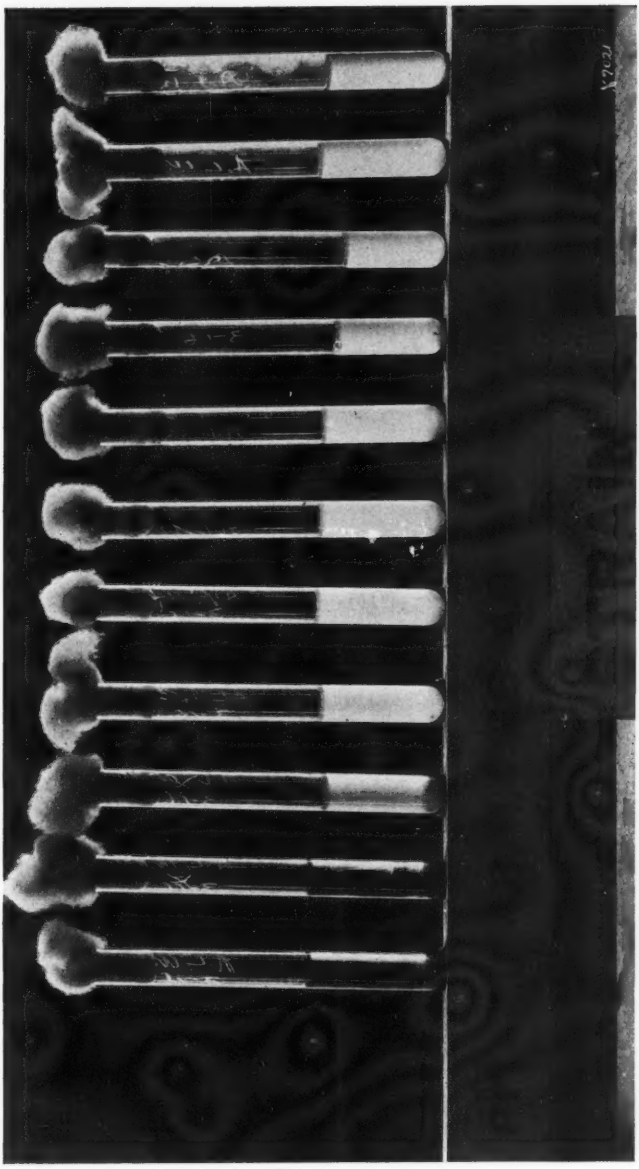

PLATE 5

CULTURES OF STRAIN 5 IN BUCHANAN'S SOLUTION AFTER 2 WEEKS AT 28°C.

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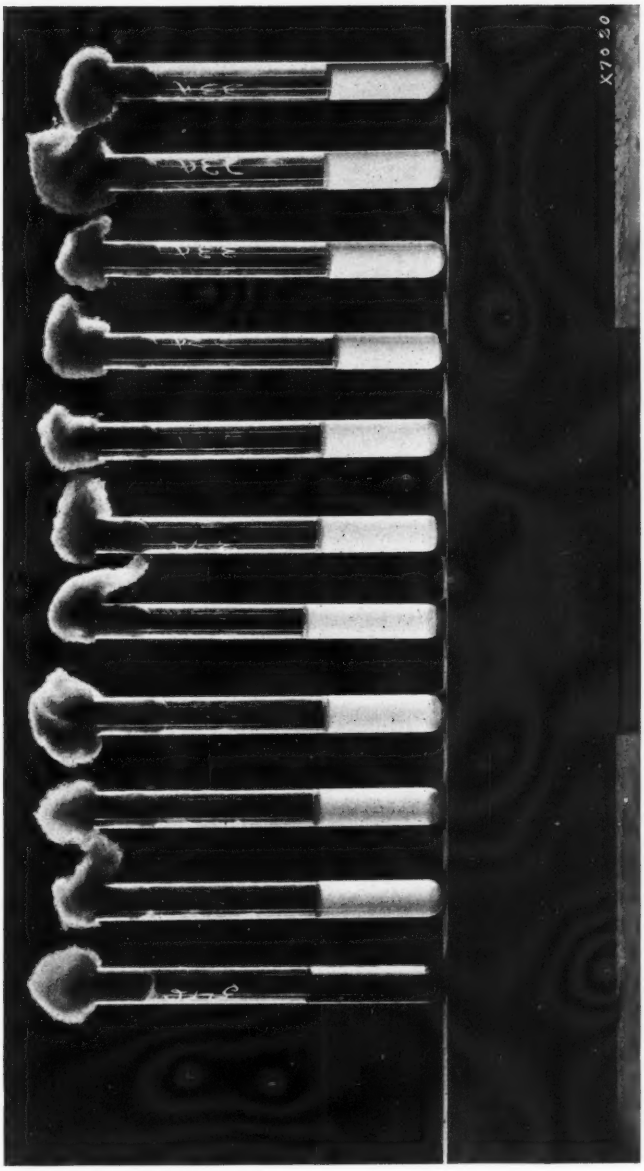

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# THE NODULE BACTERIA OF SOYBEANS: II. NITROGEN-FIXATION EXPERIMENTS[1]

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Received for publication March 27, 1925

## INTRODUCTION

In part I it was shown that all strains of *Pseudomonas radicicola* of *Soja max* are not identical. Each of these biotypes, described as type A and type B, varying about a mode of its own, must possess protoplasm differing in constitution from the other. Such disparity of the strains intimates a possibility of variation in nitrogen-fixing power.

In order to determine any difference in nitrogen-fixing power, an extended series of greenhouse and field experiments was planned.

## NITROGEN ASSIMILATION BY DIFFERENT STRAINS OF SOYBEAN NODULE BACTERIA

Greenhouse and field experiments were carried out over a period of 3 years. The results presented in this paper are for only six strains. The greenhouse trials were made with the Ito San variety of soybeans but in the field trials, Ito San, Wisconsin Black and Manchu varieties were used.

### *Greenhouse experiments*

The greenhouse experiments were made with sand cultures and Bryan's (1) modification of Crone's nutrient solution. This solution in sand cultures, without nitrogen, proved very suitable for both plant growth and nodule formation. The plants were grown in 1-gallon stone pots containing 2.5 kgm. of washed and ignited white sand, the pots, sand, and nutrient solution having been sterilized before planting. At the time of planting, enough of the nutrient solution was added to give 50 per cent saturation.

Two bacterial-free seedlings were planted in each pot and inoculated with 30 cc. of a heavy suspension of the proper strain of nodule bacteria. All tests were carried out in triplicate. Six uninoculated pots were used as controls. During the 3 years' experiments, the plants were always grown during the

[1] Part II of a thesis submitted to the Faculty of the Graduate School of the University of Wisconsin in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

months of March, April, and May. The moisture content was maintained at approximately 50 per cent of saturation with sterilized distilled water.

Each year the plants were allowed to come as close as possible to maturity without defoliation. When harvested, the number of plants, color, height, number of pods, size and number of nodules were noted. The plants were always washed from the sand in order to save the roots and nodules. After making nodule counts, and taking specimens for reisolation of cultures, the entire plants were dried and ground for analysis.

TABLE 1

*Greenhouse experiment of 1921 with six strains of soybeen nodule bacteria on Ito San soybeans*

| NUMBER OF POTS | STRAINS OF BACTERIA | HEIGHT OF PLANTS | NODULES PER PLANT | OVEN-DRY WEIGHT PER POT | NITROGEN | | |
|---|---|---|---|---|---|---|---|
| | | | | | Per gram dry weight | Per plant | Fixed |
| | | *inches* | | *gm.* | *mgm.* | *mgm.* | *mgm.* |
| 6 | None | 8 | 0 | 1.3 | 17.98 | 11.70 | −0.13 |
| 3 | 1 | 25 | 73 | 3.8 | 28.60 | 54.34 | 42.51 |
| 3 | 2 | 24 | 32 | 3.5 | 25.10 | 43.75 | 31.91 |
| 3 | 3 | 25 | 91 | 3.2 | 23.74 | 37.92 | 26.09 |
| 3 | 4 | 25 | 64 | 3.0 | 23.50 | 37.50 | 25.67 |
| 3 | 5 | 12 | 50 | 2.8 | 20.87 | 29.26 | 17.43 |
| 3 | 6 | 10 | 31 | 3.0 | 21.10 | 31.75 | 19.92 |

TABLE 2

*Number of nodules and amounts of nitrogen fixed by six strains of soybean bacteria in sand cultures*

| NUMBER OF POTS | STRAINS OF BACTERIA | NODULES PER PLANT | | | NITROGEN FIXED PER PLANT | | |
|---|---|---|---|---|---|---|---|
| | | 1921 | 1922 | 1923 | 1921 | 1922 | 1923 |
| | | | | | *mgm.* | *mgm.* | *mgm.* |
| 3 | 1 | 73 | 59 | 42 | 42.51 | 45.40 | 45.16 |
| 3 | 2 | 32 | 30 | 24 | 31.91 | 44.45 | 35.76 |
| 3 | 3 | 91 | 45 | 38 | 26.09 | 21.62 | 38.16 |
| 3 | 4 | 64 | 47 | 19 | 25.67 | 31.80 | 36.58 |
| 3 | 5 | 50 | 27 | 15 | 17.43 | 28.33 | 18.32 |
| 3 | 6 | 31 | 40 | 20 | 19.92 | 22.00 | 21.11 |

It has been shown by Whiting and Schoonover (2) that the nitrogen content of different sized seeds of the same species is variable. To take this factor into account the nitrogen content of each lot of seeds was determined, and correction made for it in calculating the nitrogen fixed.

### *Results of greenhouse experiments*

The results of the trials for each year were tabulated in order to compare the color, height, pods, nodules, dry weights, and nitrogen fixation of the plants inoculated with each strain of bacteria. In each of the three experiments the uninoculated plants were small and yellow, and failed to fix nitrogen.

The inoculated plants in every case were taller, of a dark green color, possessed numerous nodules, and fixed nitrogen. The plants inoculated with strains 5 and 6 were not so large as the plants inoculated with the other strains, and the foliage was a light green. In every case there were more pods on the inoculated plants. The dry weights of the inoculated plants were more than double those of the uninoculated plants. Some of these differences are apparent in the series of the pots in the 1923 experiment shown in plate 1.

In table 1 the detailed results of the 1921 greenhouse experiments are given. A summary of the results of 3 years is presented in table 2. It is interesting to note that the two principal types of cultures when used in greenhouse sand cultures have shown rather consistent differences in nitrogen fixed and in the

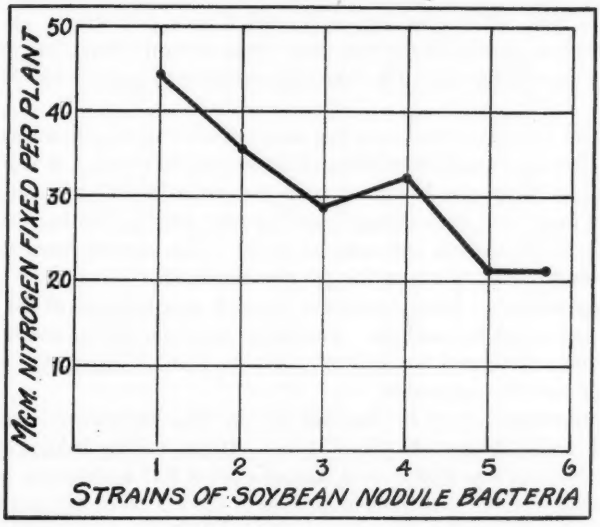


FIG. 1. GRAPHIC REPRESENTATION OF THE AVERAGE NITROGEN FIXATION OF SIX STRAINS OF SOYBEAN BACTERIA IN THE GREENHOUSE FOR THREE YEARS

yield of dry plant tissue. During the 3 years, strains 1, 2, and 3 (type A) showed an average fixation of more than one and one-half times that of strains 5 and 6 (type B). The average fixation of strain 4 was nearly the same as that of strains 1, 2 and 3. The relative amounts of nitrogen fixed, based upon the average for 3 years, is shown graphically in figure 1.

### *Field experiments*

The field experiments were made during the regular soybean seasons of 1921, 1922, and 1923. The soils selected were in different parts of the state where soybean growing is profitable. The soils were of the usual Plainfield type,

usually acid, and frequently deficient in calcium and phosphorous, as well as nitrogen.

Plots were measured off into 1/200-acre squares, 14.76 feet on each side. Cultivated, but unplanted aisles were left between the plots on all sides. Each year 36 of these plots were planted. This number provided for as many uninoculated as inoculated plots, and made possible the separation of inoculated from uninoculated plots.

In the 1921 and 1922 experiments the seed was treated with calcium hypochlorite to remove the bacteria. They were then washed in sterilized distilled water and inoculated. The seed used in the 1923 experiment was treated with mercuric chloride. In each plot 8 rows, 20 inches apart, were planted. During the growing season the plants were hand weeded and hand cultivated, and one or two final cultivations were made with a horse cultivator. The plants were allowed to grow until the first pods began to turn yellow, but not until the leaves were falling off. The time from planting to harvest was 90 to 103 days.

At harvest time the plants were dug with a spade and the soil was carefully removed in order to save the nodules of the inoculated plants. A representative lot of 50 plants was harvested from the center of each inoculated plot. The same number of uninoculated plants of each variety was harvested as a composite of the several uninoculated plots. The samples were prepared for analysis in the same way as the greenhouse plants.

*Chemical analysis.* Large samples of the soils were collected at the time of planting and saved for analysis. The plants from one pot or one field plot were ground to 200 mesh for analysis. For the Kjeldahl nitrogen determinations 1-gm. samples were used.

*Field experiments of 1921.* The soil for the 1921 experiments was a fine Plainfield sand, almost of the "blow" type. It had a water-holding capacity of 35 per cent and was slightly acid, having a pH of 6.15 as determined by the electrometric method. Analysis of samples of the soil taken at planting time gave the following results: phosphorus as $P_2O_5$, 0.025 per cent; potassium as $K_2O$, 0.854 per cent; nitrogen, 0.088 per cent; and calcium carbonate, 0.013 per cent.

The yields, dry weights, and nitrogen fixed by the three varieties of soybeans inoculated with the different strains of nodule bacteria are shown in table 3. The plants in this plot had a longer time for maturing than those of 1922 and 1923. The time from planting to harvesting was 103 days. During this time the plot received 1103 hours of sunshine and 11.3 inches of rainfall. Due to the longer growing period, the yield of dry material, and of total nitrogen, was higher than for the succeeding years. When the plants were harvested, the effect of the inoculation was very apparent. The inoculated plants of all three varieties were taller, wider leaved, more bushy, and of a darker green color.

The most important difference brought out by the data in the table is the

consistently lower yield of dry matter and of nitrogen fixed by plants inoculated with strains 5 and 6. These strains gave the lowest fixation in the field experiments.

*Field experiments of 1922.* For the 1922 experiments a somewhat heavier sandy soil was selected. This soil was a Plainfield sandy loam. It had a water-holding capacity of 42.6 per cent and was acid, having a pH value of

TABLE 3

*1921 field plot inoculation tests with six strains of soybean nodule bacteria*

50 plants were used for analysis

| STRAIN | NODULES | DRY WEIGHT | NITROGEN | TOTAL NITROGEN | NITROGEN FIXED |
|---|---|---|---|---|---|
| | | *gm.* | *per cent* | *gm.* | *gm.* |
| | | | *Wisconsin Black* | | |
| Uninoculated | 0 | 525 | 2.22 | 11.66 | 0 |
| 1 | Excellent | 995 | 3.25 | 32.34 | 20.68 |
| 2 | Excellent | 1,145 | 3.84 | 43.97 | 32.31 |
| 3 | Excellent | 1,010 | 3.64 | 36.76 | 25.10 |
| 4 | Excellent | 1,018 | 3.88 | 39.50 | 27.84 |
| 5 | Excellent | 695 | 3.00 | 20.85 | 9.79 |
| 6 | Excellent | 885 | 3.00 | 26.55 | 16.76 |
| | | | *Ito San* | | |
| Uninoculated | 0 | 785 | 2.19 | 17.19 | 0 |
| 1 | Excellent | 1,035 | 3.76 | 40.12 | 22.93 |
| 2 | Excellent | 1,300 | 3.35 | 43.55 | 25.36 |
| 3 | Excellent | 1,226 | 3.25 | 39.85 | 22.66 |
| 4 | Excellent | 1,200 | 3.41 | 40.92 | 23.73 |
| 5 | Excellent | 875 | 2.67 | 23.36 | 6.17 |
| 6 | Excellent | 950 | 2.55 | 24.23 | 7.04 |
| | | | *Manchu* | | |
| Uninoculated | 0 | 740 | 1.47 | 10.88 | 0 |
| 1 | Excellent | 1,150 | 3.30 | 37.95 | 27.07 |
| 2 | Excellent | 1,285 | 3.12 | 40.09 | 29.21 |
| 3 | Excellent | 1,260 | 2.69 | 33.89 | 23.01 |
| 4 | Excellent | 1,120 | 3.86 | 42.23 | 31.35 |
| 5 | Excellent | 980 | 2.50 | 24.50 | 13.62 |
| 6 | Excellent | 900 | 2.28 | 20.52 | 9.64 |

5.04. Analysis of samples at planting time showed the following composition: phosphorous as $P_2O_5$, 0.038 per cent; potassium as $K_2O$, 0.724 per cent; nitrogen, 0.084 per cent; and organic matter, 1.36 per cent.

The yields and dry weights were determined as in the preceding year. The growing period for these plants was shorter than that of the first year, being 90 days. The plants were not so mature, giving a lower yield of dry matter

and less nitrogen fixation. During the growing period they received 788 hours of sunshine and 11.2 inches of rainfall.

The difference in color of the inoculated and uninoculated plants was not so distinct as in the previous year. However the larger size and bushiness of the inoculated plants was apparent. The results as given in table 4 show that the inoculated plants produced more dry weight and contained a higher percentage of nitrogen in every case.

TABLE 4

*1922 field plot inoculation tests with six strains of soybean nodule bacteria*

50 plants were used for analysis

| STRAIN | NODULES | DRY WEIGHT | NITROGEN | TOTAL NITROGEN | NITROGEN FIXED |
|---|---|---|---|---|---|
| | | *gm.* | *per cent* | *gm.* | *gm.* |
| | | *Wisconsin Black* | | | |
| Uninoculated | 0 | 690 | 2.66 | 18.35 | 0 |
| 1 | Excellent | 966 | 3.08 | 29.75 | 11.40 |
| 2 | Excellent | 1,294 | 3.28 | 41.54 | 23.19 |
| 3 | Excellent | 965 | 2.88 | 27.79 | 9.44 |
| 4 | Excellent | 992 | 3.06 | 30.36 | 12.01 |
| 5 | Good | 724 | 2.74 | 19.84 | 8.43 |
| 6 | Good | 768 | 2.84 | 21.81 | 3.48 |
| | | *Ito San* | | | |
| Uninoculated | 0 | 716 | 2.67 | 19.12 | 0 |
| 1 | Excellent | 980 | 2.95 | 28.91 | 9.79 |
| 2 | Excellent | 1,195 | 3.23 | 38.60 | 19.48 |
| 3 | Excellent | 920 | 2.95 | 27.14 | 8.02 |
| 4 | Excellent | 1,016 | 3.25 | 33.02 | 13.90 |
| 5 | Good | 714 | 2.90 | 20.71 | 1.59 |
| 6 | Good | 755 | 2.95 | 22.27 | 3.15 |
| | | *Manchu* | | | |
| Uninoculated | 0 | 580 | 2.66 | 15.43 | 0 |
| 1 | Good | 1,018 | 3.06 | 31.15 | 15.72 |
| 2 | Good | 1,112 | 2.71 | 19.04 | 3.60 |
| 3 | Good | 902 | 2.88 | 27.98 | 12.55 |
| 4 | Good | 1,220 | 3.15 | 38.43 | 23.00 |
| 5 | Fair | 983 | 2.09 | 20.55 | 5.12 |
| 6 | Fair | 641 | 2.79 | 17.88 | 2.45 |

Comparisons of the yields of dry material and the amounts of nitrogen fixed show more variation among the strains than in 1921. Strains 5 and 6 are consistently lower than all the others with the single exception of strain 2 on Manchu soybeans. Here, on a somewhat more favorable soil type with practically the same weather conditions, these strains gave lower fixation than the others.

*Field experiments of 1923.* A very acid Plainfield sand was selected for the 1923 experiments. This soil had a water-holding capacity of 30.1 per cent and a pH value of 4.42. Analysis of the composite sample, taken when the plots were planted, gave the following results: phosphorous as $P_2O_5$, 0.032 per cent; potassium as $K_2O$, 1.086 per cent; nitrogen, 0.068 per cent; calcium carbonate, 0.021 per cent; and organic matter, 0.620 per cent. The growing period was

TABLE 5

*1923 field plot inoculation tests wih six strains of soybean nodule bacteria*

50 plants were used for analysis

| STRAIN | NODULES | DRY WEIGHT | NITROGEN | TOTAL NITROGEN | NITROGEN FIXED |
|---|---|---|---|---|---|
| | | *gm.* | *per cent* | *gm.* | *gm.* |
| | | *Wisconsin Black* | | | |
| Uninoculated | 0 | 262 | 1.80 | 4.72 | 0 |
| 1 | Excellent | 491 | 2.27 | 11.15 | 6.43 |
| 2 | Good | 458 | 2.98 | 13.75 | 9.03 |
| 3 | Excellent | 645 | 2.86 | 18.44 | 13.72 |
| 4 | Excellent | 354 | 2.85 | 10.09 | 5.37 |
| 5 | Excellent | 375 | 2.04 | 7.65 | 2.93 |
| 6 | Excellent | 548 | 2.38 | 13.04 | 8.38 |
| | | *Ito San* | | | |
| Uninoculated | 0 | 254 | 1.66 | 4.21 | 0 |
| 1 | Good | 667 | 2.10 | 14.00 | 9.79 |
| 2 | Good | 390 | 2.55 | 9.95 | 5.74 |
| 3 | Good | 467 | 2.98 | 13.92 | 9.71 |
| 4 | Good | 450 | 2.84 | 12.78 | 8.57 |
| 5 | Excellent | 390 | 1.84 | 6.18 | 1.97 |
| 6 | Excellent | 489 | 1.89 | 9.24 | 5.03 |
| | | *Manchu* | | | |
| Uninoculated | 0 | 237 | 1.97 | 4.67 | 0 |
| 1 | Good | 390 | 2.47 | 9.63 | 4.94 |
| 2 | Very Poor | 260 | 2.04 | 5.30 | 0.63 |
| 3 | Poor | 340 | 2.94 | 9.10 | 4.43 |
| 4 | Fair | 245 | 2.59 | 6.35 | 1.68 |
| 5 | Good | 332 | 2.12 | 7.04 | 2.37 |
| 6 | Excellent | 325 | 2.21 | 7.18 | 2.51 |

96 days. During this time there were 11.03 inches of rainfall and 814 hours of sunshine.

This plot was on top of a well-drained, sandy hill which undoubtedly had quite a marked effect upon the moisture supply of the plants. The dry-weight yields of all of the plots were lower than in the previous years. Notwithstanding this fact all the inoculated plants showed an increased dry weight and nitrogen content as shown in table 5.

## DISCUSSION

An extensive series of laboratory, greenhouse and field studies was made with six strains of soybean nodule bacteria on three varieties of soybeans. In addition to morphological, cultural, physiological and serological studies of the strains, their nitrogen-fixing power was studied. The nitrogen assimilation of three varieties of soybeans treated with six strains of nodule bacteria was determined.

Strains 5 and 6 (type B) consistently brought about a lower fixation of nitrogen for 3 years on all three varieties of soybeans (table 6). The Wisconsin

TABLE 6

*Summary of three years' field inoculation of three varieties of soybeans with six strains of bacteria*

In every case 50 plants were used for analysis

| STRAINS | WISCONSIN BLACK | | ITO SAN | | MANCHU | |
|---|---|---|---|---|---|---|
| | Dry weight | Nitrogen fixed | Dry weight | Nitrogen fixed | Dry weight | Nitrogen fixed |
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| 1 | 815 | 12.84 | 894 | 14.07 | 853 | 15.91 |
| 2 | 965 | 21.51 | 961 | 16.86 | 886 | 11.41 |
| 3 | 873 | 16.08 | 871 | 13.46 | 834 | 13.33 |
| 4 | 788 | 15.07 | 888 | 15.40 | 861 | 15.31 |
| 5 | 598 | 7.05 | 659 | 4.48 | 765 | 7.04 |
| 6 | 734 | 9.54 | 731 | 5.07 | 622 | 4.87 |

TABLE 7

*Three years' average fixation and dry weight yield of all plants inoculated with six strains of soybean nodule bacteria*

50 plants were used for analysis

| STRAINS | DRY WEIGHTS | NITROGEN FIXED |
|---|---|---|
| | *gm.* | *gm.* |
| 1 | 854 | 14.31 |
| 2 | 870 | 16.50 |
| 3 | 866 | 14.29 |
| 4 | 846 | 15.19 |
| 5 | 674 | 6.19 |
| 6 | 696 | 6.49 |

Black variety received the most benefit from inoculation. The nitrogen fixation of this variety, when inoculated with the high nitrogen-fixing (type A) strains, was higher on a 3-year average than Ito San or Manchu. The nitrogen-fixing power of Ito San was greater than Manchu with the type A strains.

The 3-year average for all strains of nodule bacteria on three varieties of soybeans is given in table 7. Including the variable results of 1923, there is remarkable agreement between the numbers of the low and high nitrogen-fixing strains. Such differences are too marked and consistent to be due to

environmental factors or to chance. This is especially important when the cultural and physiological differences between strains 5 and 6 and the others are considered.

The correlations of the data appear to show two types of soybean nodule bacteria, one capable of the most decided beneficial symbiosis with the green plant, the other benefiting the plant much less.

Many years ago, Hiltner writing in Lafar's "Handbuch der Technischen Mykologie," volume 3, page 45, set forth six conditions as instances in which the legume plant might resist the nodule bacteria. Two of these cases, (the third) "the organisms enter the plant and no fixation of nitrogen occurs" and (the fifth) "the organisms are parasitic, affecting the plant but little" might help to explain the results with the low nitrogen-fixing strains.

## CONCLUSIONS

1. Different varieties of *Soja max* do not receive the same benefit when inoculated with a type B strain of *Pseudomonas radicocola* as when inoculated with a type A strain.

2. As shown by 3 years' tests, in greenhouse and field, plants inoculated with type A strains fixed more nitrogen than plants inoculated with strains of type B.

3. The size, number, and distribution of nodules produced by type A are different from type B. These differences are more apparent when the seed is inoculated than when the bacteria are mixed in the soil.

4. The fact that the type A strains fixed more than twice as much nitrogen as the type B strains is a matter of considerable importance in practical soybean culture.

5. The observations reported in this paper and in part I show two distinct varying units, biotypes, in the six strains of nodule bacteria studied. Although considerable variability occurs among the legume bacteria, the differences between these types have persisted throughout the changes in the numerous environmental factors.

If these types through some of their physiologic characteristics have become separated in nature, there may be other biotypes that have not been isolated.

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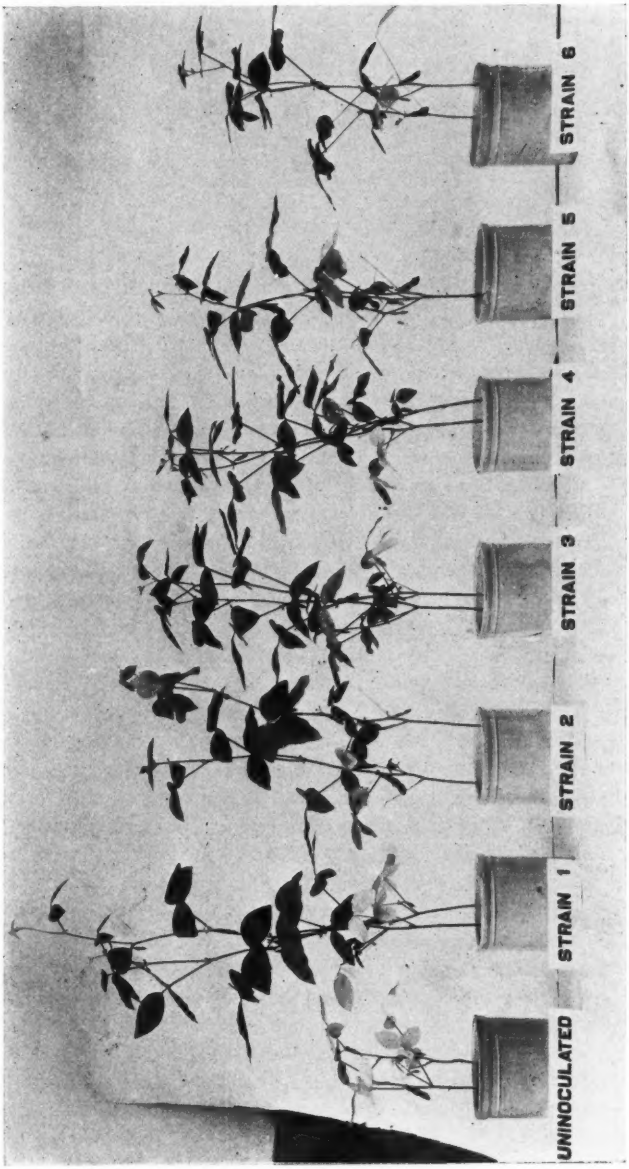
## PLATE 1

A Series of Pots from the 1923 Greenhouse Experiment Showing the Appearance of the Uninoculated Plants and Those Inoculated with Different Strains of Soybean Bacteria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STRAIN 4
STRAIN 5
STRAIN 6

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# MODIFYING RHIZOBIUM RADICICOLUM

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Received for publication March 31, 1925

Changes in the characteristics of pure bacterial cultures are generally brought about either by exposure to a new environment or by the dissociation of the culture into its different strains. Recently a third method, that of cross breeding, has been suggested (1).

Changing the characteristics of a culture by exposure to a new environment is practiced in increasing pathogenicity by animal passage. Conversely, growing a freshly isolated pathogenic organism on artificial media results in a loss of virulence. Modifying a culture by separating the strains composing it is not commonly practiced. Churchman (2) has shown that a culture of *Bact. coli* may contain two strains, differing only in that one strain is more resistant to gentian violet. By the process of selection the two strains were separated and the difference in dye resistance was found to be transmitted to subsequent transplants. De Kruif (3) has demonstrated that cultures of the bacillus of rabbit septicemia may contain individuals varying in degree of virulence, and that this difference is retained by transplants made from colonies arising from such individuals. The work of Churchman and De Kruif indicates that cultures of bacteria considered as pure by accepted standards may consist of individuals not identical in character. Selection and separation of the individuals that differ result in a modification of the culture.

In animal passage the process of selection may be the main factor modifying the culture, since only those cells that are able to grow in the body survive and are recovered and used in subsequent injections. It is doubtful, however, if selection accounts for all the change occurring in a culture when exposed to a new environment. There is evidence to indicate that the cells of such a culture may become modified sufficiently to differ markedly from any one of the cells of the original culture. Adaptation of a culture to a new environment may occur as the result of both selection and modification. The possibility of mutations occurring must also be considered.

Little attempt has been made to improve cultures of *R. radicicolum* either by the process of dissociation into desirable and undesirable strains within a culture or by exposure to a changed environment. Modifying the cultures to meet special soil conditions may prove advantageous. Furthermore, if *R. radicicolum* becomes modified when subjected to changed conditions it may lose some desirable characteristics when grown for long periods on media.

The common laboratory practice of sending out cultures from old laboratory strains for legume inoculation may not be the most effective. In the present paper are described experiments demonstrating that *R. radicicolum* may become modified when subjected to a new environment.

### EXPERIMENT 1. TO DETERMINE WHETHER R. RADICICOLUM CAN INCREASE ITS ABILITY TO TOLERATE GENTIAN VIOLET

*Technique.* A freshly isolated strain of *R. radicicolum* was stroked for several generations on 1:10,000 gentian violet agar. This is approximately the greatest concentration of the dye in which freshly isolated strains will

TABLE 1

*Results of experiment to determine whether R. radicicolum can increase its tolerance for gentian violet**

| | DILUTION OF DYE IN AGAR | | | | | |
|---|---|---|---|---|---|---|
| | 1:10,000 | 1:5,500 | 1:4,000 | 1:3,000 | 1:1,500 | 1:1,000 |
| Original culture | + | | | | | |
| Dye-tolerant culture | +++ | +++ | +++ | ++ | ++ | + |

*+++ indicates as heavy growth on the dye agar as on the dye-free agar. ++ and + indicate a proportionate reduction in growth on the dye agar.

TABLE 2

*Results of experiment to determine whether a dye-tolerant culture of R. radicicolum will lose its tolerance for gentian violet when grown on dye-free agar**

| | DILUTION OF DYE IN AGAR | | | | | |
|---|---|---|---|---|---|---|
| | 1:10,000 | 1:5,500 | 1:4,000 | 1:3,000 | 1:1,500 | 1:1,000 |
| Dye-tolerant culture grown on dye-free agar for 3 generations | ++ | | | | | |
| Dye-tolerant culture | +++ | +++ | ++ | ++ | + | ++ |

*+++ indicates as heavy growth on the dye agar as on the dye-free agar. ++ and + indicate a proportionate reduction in growth on the dye agar.

grow. This culture and the original cultures were then stroked on agar plates, half of each plate containing gentian violet agar and the other half dye-free agar. The concentration of gentian violet in the agar varied from 1:10,000 to 1:1000. The reaction of the agar before the dye was added varied from pH 6.9 to pH 7.1.

The results obtained are given in table 1 and indicate that *R. radicicolum* can increase its ability to grow in the presence of gentian violet. The culture which had been grown for several generations on a 1:10,000 gentian violet agar grew as well on 1:1000 gentian violet agar as the original culture grew on 1:10,000 gentian violet agar (fig. 1).

### EXPERIMENT 2. TO DETERMINE WHETHER THE INCREASED TOLERANCE FOR GENTIAN VIOLET REPRESENTS A PERMANENT CHANGE IN THE CULTURE

*Technique.* The dye-tolerant culture obtained in experiment 1 was stroked for 3 generations on dye-free agar. This culture and the original dye-tolerant culture were stroked on dye agar as in experiment 1.

The results obtained are given in table 2 and indicate that the increased tolerance for dye is quickly lost when the organisms are grown on dye-free agar. With a change in the environment, *R. radicicolum* may gain or lose the ability to grow in the presence of gentian violet in certain concentrations (fig. 2).

The dye-tolerant cultures were found to produce nodules on legume rootlets.

## DISCUSSION

Our experiments demonstrate that a culture of *Rhizobium radicicolum* can increase its ability to grow under unfavorable conditions and that when the unfavorable conditions are removed it quickly loses that power. It may modify a character relatively quickly. This fact is of considerable theoretical importance. The possibility of *R. radicicolum* losing a desirable characteristic when grown on artificial media should be determined. The accepted method of keeping cultures of *R. radicicolum* may not prove to be the most effective for producing cultures for soil inoculation. While our experiments suggest that *R. radicicolum* may, when returned to the soil, quickly regain any character it has lost as the result of being grown on media, the possibility of a less effective inoculation must be considered until other evidence is presented. It may prove desirable to give the organisms an opportunity to adapt themselves to the type of soil to be seeded. The evidence presented here favors the claims made by others that it is possible by subjecting the organisms to a suitable environment to vary their virulence.

The modification of laboratory strains of *R. radicicolum* to meet special soil conditions is worthy of consideration. If such modification results in a permanent change, the propagation of *R. radicicolum* for inoculation is relatively simple. If, however, the modification is only temporary and lost as soon as the environment changes, the problem is more difficult. The changes produced by our experiments were temporary but in view of what has been done in the dissociation of species it is conceivable that permanent changes also may take place. The fact that a bacterial culture may consist of individual cells which differ materially and that one type may crowd out the other, as has been demonstrated for *Neisseria catarrhalis*, must be considered (4). It suggests that a culture may be improved or deteriorated by the selection of one type of cell in preference to the other. Cultures of *R. radicicolum* may, when left for indefinite periods on media, deteriorate not only because of a loss or reduction in degree of some character but also because

the culture may contain two strains or types, one less desirable which may crowd out the desirable type. The reverse may also occur. Furthermore, an occasional plating out to remove contaminants or determine purity may result in the selection of either strain.

In the isolation of *R. radicicolum* from root nodules it has been generally assumed that the cells giving rise to colonies on the plates have the same potentialities and that the selection of any one for stock cultures will prove as satisfactory as any other. In the light of recent advances in the knowledge of the plurality of strains within a culture, this assumption is not altogether justified. Until much research has been done and the point determined it seems advisable to make up the stock cultures from a large number of colonies.

The modification of bacterial cultures for definite purposes by the processes of exposure and selection or dissociation offers a new and promising field for experimentation. Whether modification by exposure to changed environmental conditions or the dissociation of strains within a culture offers the more promising method for improving cultures of *R. radicicolum* remains to be determined.

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(3) De Kruif, Paul H. 1921 Dissociation of microbic species. Coexistence of individuals of different degrees of virulence in cultures of the bacillus of rabbit septicemia. *In* Jour. Exp. Med., v. 33, p. 77.

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Fig. 1. Plate with Gentian Violet Agar on One Side and Dye-free Agar on the Other Side

The upper stroke was made from a culture of *R. radicicolum* grown for several generations on gentian violet agar and the lower stroke from the stock culture.

Fig. 2. Plate Used Similar to Plate Shown in Figure 1

The upper stroke is from a dye-tolerant culture of *R. radicicolum* and the lower stroke from the same strain after growing for three generations on dye-free agar.

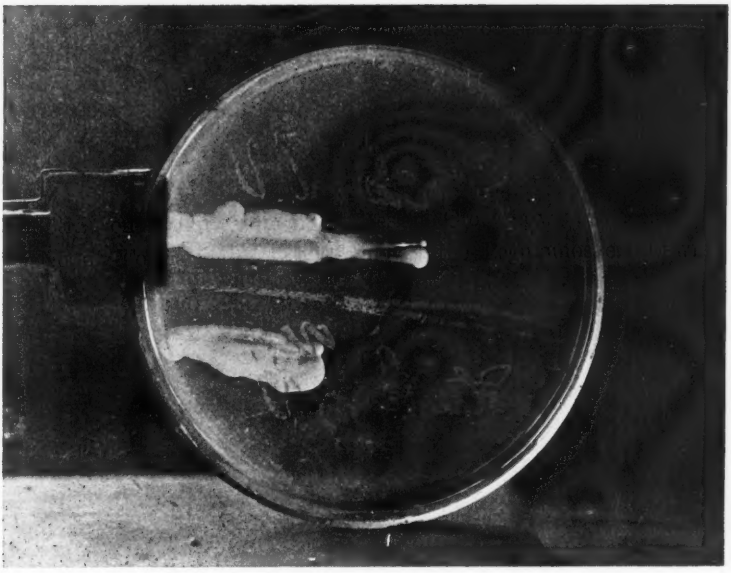

FIG. 1

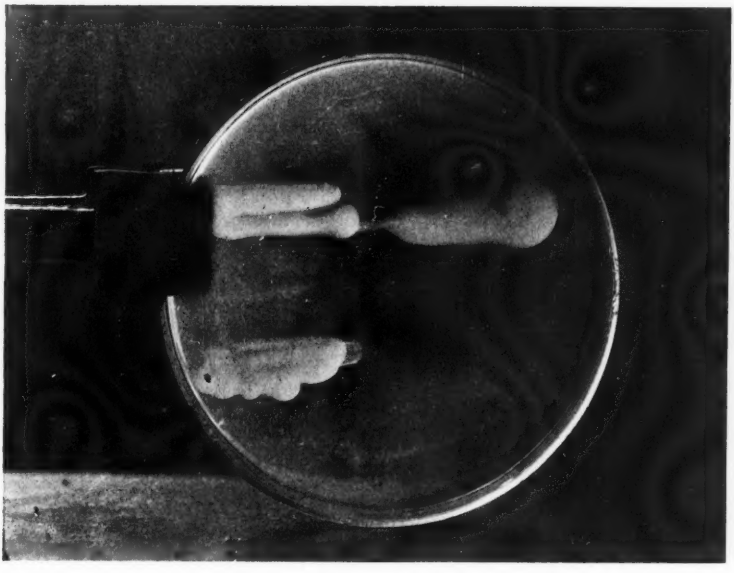

FIG. 2

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# THE ABSORPTION OF PHOSPHATE BY PASTEUR-CHAMBERLAND FILTERS

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Received for publication April 13, 1925

While making a study of the phosphorus content of soil extracts, the writer obtained results that indicated an error in the method of preparing the extracts. A study of the possible sources of error was made and it was found that the phosphate content of the extracts was altered during filtration through Pasteur-Chamberland filters. Additional studies were then made to determine (*a*) the amount of phosphate that may be absorbed by the filter from solutions of known concentration; (*b*) how readily the absorbed phosphate may be removed by washing; and (*c*) the cause of the absorption and if possible some method to prevent it. The results of these studies are presented in this paper.

The only data found in the literature bearing on the subject is that of Stewart (3, p. 327). Stewart determined the phosphate content of known phosphate solutions before and after filtering through Pasteur-Chamberland filters. In every instance he found a lower concentration of phosphorus in the filtered solution than in the unfiltered solution. He apparently did not consider the results significant, as no mention was made of these differences in discussing his data. This was probably due to the fact that the method of determining phosphorus was considered unsatisfactory. In all of his subsequent work Stewart used an entirely different method for determining phosphorus but he did not repeat the work on the influence of filtering on the phosphate content of the solutions.

## METHODS FOR PHOSPHORUS DETERMINATION

In studying the magnitude of absorption by the filters it is essential that the method used for determining phosphorus be accurate and sufficiently sensitive to make possible the estimation of very small amounts of phosphorus. The Coeruleo-molybdate method of Deniges (2) has been used in this investigation. Atkins (1) used this method in a study of the phosphate content of soil extracts and presented some data indicating its accuracy. Before adopting the method for use in this laboratory the procedure of Atkins was modified in several respects. The procedure recommended and used in this laboratory is as follows:

Place 100 cc. of the soil solution or extract in a 150-cc. casserole, add 1 cc. of 1:1 $HNO_3$ and evaporate to dryness on the water bath. Ignite to destroy organic matter. After cooling add 5 cc. of a 5 per cent HCl solution and place on the boiling water bath for 5 minutes. Work the acid over the inside of the casserole to bring all phosphorus into solution. After removing from the water bath transfer to a 100-cc. graduated cylinder, washing the casserole with distilled water. If the original solution contains less than 0.3 p.p.m. $PO_4$, dilute to 97.5 cc. If it contains more than 0.3 p.p.m. dilute to 100 cc., then take an aliquot part of the 100 cc. and dilute it to 97.5 cc. For the most satisfactory results this solution should not contain more than 0.3 p.p.m. $PO_4$. Transfer the solution to a 150-cc. Erlenmeyer flask.

To develop the blue color add 2 cc. of reagent A and 5 drops of reagent B. Agitate the solution while adding reagent B. Allow the solution to stand about 10 minutes before comparing with the standard of approximately the same concentrations as the unknown. The color comparison is conveniently made with Nessler tubes.

Reagent A is made by mixing 100 cc. of a 10 per cent solution of ammonium molybdate with 300 cc. of a 50 per cent (by volume) solution of $H_2SO_4$. This solution should be stored in the dark.

Reagent B is prepared fresh each day. To 0.5 gm. of powered tin add 5 drops of a 4 per cent $CuSO_4$ solution and 10 cc. conc. HCl, free of arsenic. Warm to hasten the reaction and finally dilute to 50 cc.

The standards are prepared by using 5 to 30 cc. of a known phosphate solution containing 1.0 p.p.m. $PO_4$. The solution is diluted to about 95 cc., the color developed and then diluted to exactly 100 cc.

If the solution in which phosphorus is to be determined is perfectly clear, not colored with organic matter, it will not be necessary to evaporate and ignite before making the determination. Most of the determinations reported in this paper were made without evaporating the solution.

After the procedure was standardized the results obtained by the method were compared with those obtained by the standard volumetric method. For this purpose large quantities of 1:5 soil extracts were prepared and the concentration of phosphorus determined by the two methods. The extracts were prepared from the soil of a series of plots which had received different amounts of acid phosphate, applied to cotton, 7 months before sampling. For the volumetric determination 10 to 13 liters of extract were evaporated. This determination was not in duplicate. The colorimetric determinations were in duplicate, the procedure given above being used. Table 1 gives the results obtained by the two methods.

The results obtained by the two methods agree remarkably well. The experiment was repeated using three other soils and in every case the agreement was very good.

To test further the colorimetric method the following solutions were prepared:

A. Soil extract from a sandy soil.
B. 1000 cc. of A plus 1000 cc. of distilled water.
C. 1000 cc. of A plus 1000 cc. of a known phosphate solution containing 0.634 p.p.m. $PO_4$.

The $PO_4$ content of the above solutions was then determined by the colorimetric method. The results are given in table 2. The agreement between the

calculated and experimental results is very good. Atkins reported a similar agreement. These results show that the method is accurate and makes possible the determination of smaller quantities of phosphate than can be determined with other methods commonly used. It has also the advantage of being short and none of the salts ordinarily found in the soil solution interferes with the reaction.

TABLE 1

*Phosphate concentration of 1:5 extracts as determined volumetrically and colorimetrically*

| FERTILIZATION PER ACRE WITH ACID PHOSPHATE | $PO_4$ IN SOIL EXTRACT | |
|---|---|---|
| | Volumetric method | Colorimetric method |
| *lbs.* | *p.p.m.* | *p.p.m.* |
| None | 0.059 | 0.056 |
| 300 | 0.102 | 0.084 |
| 600 | 0.188 | 0.190 |
| 900 | 0.442 | 0.449 |
| 1,200 | 0.643 | 0.636 |

TABLE 2

*The $PO_4$-content of known solutions as determined by the Coeruloe-molybdate method*

| | SOLUTIONS | $PO_4$ IN SOLUTION | |
|---|---|---|---|
| | | Calculated | Found |
| | | *p.p.m.* | *p.p.m.* |
| A | Soil extract | ..... | 0.225 |
| B | Diluted soil extract | 0.112 | 0.110 |
| C | Soil extract plus $PO_4$ | 0.434 | 0.444 |

ABSORPTION OF PHOSPHATES BY USED PASTEUR-CHAMBERLAND FILTERS

The study of phosphate absorption was made with 11 Pasteur-Chamberland filters that had been used in preparing soil extracts. In using them for that purpose the filters were always washed with about 500 cc. of distilled water before filtering the suspension and frequently they were dried in an oven at 110°C. to prevent bacterial action. It was found that if the filters were washed, dried at 110°C. and washed again the first portion of the last washing was rather high in phosphate. This can be illustrated by the results obtained with filters 9, 10, and 11. These filters were washed with 500 cc. of distilled water and dried in an oven at 110°C. for 48 hours. They were then washed with distilled water, and the phosphorus content of successive 100-cc. portions was determined colorimetrically. The results are given in table 3. They show that the filters give up considerable amounts of the absorbed phosphate on prolonged washing. These amounts might not be important in studying some soils. However, in a study of the phosphate content of extracts from 24 coastal plain soils the writer has found a maximum concentration of 0.74

p.p.m.$PO_4$ and a minimum concentration of 0.02 p.p.m. Of the 24 soils 19 have an extract containing less than 0.1 p.p.m. $PO_4$.

In order to study the absorption of phosphate from solution of known concentration, filters 1 to 4 were used. But before the absorption experiments were performed a study was made to determine how much phosporus was held in the filters. These filters had been used for some time in preparing soil extracts. They were alternately washed and dried at 110° four successive times, being washed with 600 to 700 cc. of water each time. The fourth time, phosphate was determined in successive 100-cc. portions with the results given in table 4.

TABLE 3

*Phosphate content of successive 100-cc. portions of washings from old Pasteur-Chamberland filters*

| PORTION | $PO_4$ IN SOLUTION | | |
|---|---|---|---|
| | Filter 9 | Filter 10 | Filter 11 |
| | *p.p.m.* | *p.p.m.* | *p.p.m.* |
| 1 | 0.150 | 0.140 | 0.170 |
| 2 | 0.100 | 0.110 | 0.124 |
| 3 | 0.075 | 0.075 | 0.072 |
| 5 | 0.052 | ..... | 0.039 |
| 15 | 0.030 | 0.030 | 0.030 |

TABLE 4

*Phosphate concentration of successive 100-cc. portions of the fourth washing of filters 1 to 4*

| PORTION | $PO_4$ IN SOLUTION | | | |
|---|---|---|---|---|
| | Filter 1 | Filter 2 | Filter 3 | Filter 4 |
| | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* |
| 1 | 0.258 | 0.210 | 0.198 | 0.276 |
| 2 | 0.073 | 0.055 | 0.060 | 0.073 |
| 3 | 0.042 | 0.032 | 0.035 | 0.042 |
| 6 | | Trace estimated at 0.015 | | |

These filters were then washed with 1500 cc. of boiling water. The hot water passed through the filters at the rate of about 250 cc. per minute. Phosphorus was determined in successive 100-cc. portions. Table 5 gives the data secured. It is evident that hot water is much more effective than cold water for washing to remove absorbed phosphate.

These filters were next used for a study of the absorption of phosphate from solutions of known concentration. Filters 1 and 2 were used in studying absorption from a potassium acid phosphate solution containing 0.2 p.p.m. $PO_4$. Filters 3 and 4 were used in studying absorption from a solution containing 1.0 p.p.m. $PO_4$. Higher concentrations of $PO_4$ were not used, since our studies indicated that southern soils rarely contain over 1.0 p.p.m. $PO_4$ in the

soil extract. Through the filters 1500 cc. of the above solutions were passed at the rate of about 100 cc. per minute. Phosphorus was determined in successive 250-cc. portions. The results are given in table 6.

The data in table 6 show that from solutions of low phosphate concentration the filters absorb enough phosphorus to make results by the usual procedure of little value. For instance, assume that one has 500 cc. of extract from a soil and the extract contains 0.2 p.p.m. $PO_4$. If the extract passed these filters it would contain approximately 0.035 p.p.m. $PO_4$, assuming that the first 100 cc. of filtrate was discarded. When solutions of high phosphate content are used

TABLE 5

*Phosphate content of successive 100-cc. portions of filtrate from filters 1 to 4. Washing with hot water*

| PORTION | $PO_4$ IN SOLUTION | | | |
|---|---|---|---|---|
| | Filter 1 | Filter 2 | Filter 3 | Filter 4 |
| | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* |
| 1 | 0.320 | 0.240 | 0.240 | 0.368 |
| 3 | 0.164 | 0.156 | 0.152 | 0.184 |
| 5 | 0.150 | 0.104 | 0.104 | 0.140 |
| 12 | 0.060 | 0.050 | 0.050 | ..... |

TABLE 6

*Phosphate content of successive 250-cc. portions of phosphate solutions after passing through used Pasteur-Chamberland filters*

| PORTION | $PO_4$ IN SOLUTION | | | |
|---|---|---|---|---|
| | Filter 1 | Filter 2 | Filter 3 | Filter 4 |
| | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* |
| 1 | 0.015 | 0.015 | 0.353 | 0.336 |
| 2 | 0.043 | 0.050 | 0.860 | 0.880 |
| 3 | 0.105 | 0.101 | 0.840 | 0.880 |
| 4 | 0.107 | 0.110 | 0.860 | 0.860 |
| 5 | 0.130 | 0.130 | 0.860 | 0.880 |
| 6 | 0.134 | 0.133 | 0.880 | 0.880 |
| Unfiltered solution | 0.200 | 0.200 | 1.000 | 1.000 |

the error is considerably reduced. This is shown by the results obtained with the solution containing 1.0 p.p.m. $PO_4$. However, even in this case the error would be large if a relatively small quantity of extract was secured. Frequently only 500 cc. or 700 cc. of extract is obtained. If that is the case the error would be large even with the higher concentrations of phosphorus.

The one factor that would tend to make the error greater in the case of soil extracts than in the case of the solutions studied, is the rate of filtration which would determine the time during which absorption may take place. In the experiment reported here the solution passed the filter at the rate of approxi-

mately 100 cc. per minute. This is many times more rapid than is frequently the case with soil extracts. Consequently absorption may be more complete from soil extracts than from phosphate solutions of the same concentration.

Filters 1 to 4 were next used to study absorption of phosphate from a 1:5 soil extract. The filters were first washed with 1500 cc. of distilled water. Filters 1 and 3 were used to filter the extract from a soil relatively low in water-soluble phosphorus. Filters 2 and 4 were used to filter the extract from a soil that had been well fertilized with acid phosphate. After preparing the soil suspension a portion was centrifuged until practically clear. The remainder was filtered. Phosphorus was determined in three portions of the filtrate; viz., the first 100 cc. that is usually discarded, the next 250 cc., and another portion of 250 cc. The results are shown in table 7. Absorption from the soil extracts was about the same as from the known phosphate solutions. It is very evident that absorption of phosphate by Pasteur-Chamberland filters is an important factor to be considered in soil studies. It may be the source of considerable error when the usual procedure is followed.

TABLE 7

*Phosphate content of filtered and centrifuged soil extract*

| PORTION | VOLUME | $PO_4$ IN SOLUTION | | | | | |
|---|---|---|---|---|---|---|---|
| | | Soil 1 | | | Soil 2 | | |
| | | Filter 1 | Filter 3 | Average | Filter 2 | Filter 4 | Average |
| | *cc.* | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* | *p.p.m.* |
| 1 | 100 | 0.025 | 0.032 | 0.028 | 0.017 | 0.025 | 0.021 |
| 2 | 250 | 0.025 | 0.027 | 0.026 | 0.232 | 0.272 | 0.252 |
| 3 | 250 | 0.039 | 0.048 | 0.044 | 0.480 | 0.512 | 0.496 |
| Centrifuged | | (1) 0.090 | (2) 0.090 | 0.090 | (1) 0.576 | (2) 0.624 | 0.600 |

ABSORPTION OF PHOSPHATE BY NEW AND IGNITED FILTERS

There are at least two possible causes for the absorption of phosphate by Pasteur-Chamberland filters that have been used in preparing soil extracts. First, absorption may be due to the filter itself. If that is the cause, new filters would have a considerable absorptive capacity. Second, absorption may be due to soil colloids held in the pores of the filter. If the soil colloids are the only cause of absorption new filters would not absorb phosphorus from a phosphate solution.

Four new filters were used to determine whether or not absorption was due to the filter itself or to the soil colloids. The 4 filters were first washed with distilled water, and the phosphate was determined in successive 100-cc. portions of the washings. The average results for the 4 filters were as follows: The first portion contained 0.392 p.p.m. $PO_4$, the second 100 cc. contained 0.167 p.p.m. $PO_4$. These filters were then washed with hot water and the phosphate content of successive 100-cc. portions was determined. The average results

were 0.94 p.p.m. $PO_4$ in the first portion, 0.697 in the second, 0.468 in the third, and 0.405 in the fourth 100-cc. portion of the filtrate. These results show that new filters contain very considerable amounts of water-soluble phosphorus. The wash water from these filters contained more phosphorus than the extract from a large number of soils.

The absorptive power of the new filters was determined with solutions containing 0.2 and 1.0 p.p.m. $PO_4$. Phosphorus was determined in successive 250-cc. portions of the filtrate. The results are presented in table 8. These

TABLE 8

*Phosphate content of known phosphate solutions after passing through new Pasteur-Chamberland filters*

| PORTION | $PO_4$ IN SOLUTION | |
|---|---|---|
| | Solution containing 0.2 p.p.m. $PO_4$ | Solution containing 1.0 p.p.m. $PO_4$ |
| | *p.p.m.* | *p.p.m.* |
| 1 | 0.264 | 0.760 |
| 2 | 0.234 | 0.940 |
| 3 | 0.230 | 0.980 |
| 4 | 0.210 | 1.020 |
| 5 | 0.192 | 0.980 |
| 6 | 0.198 | 1.020 |

TABLE 9

*Phosphorus content of successive 250-cc. portions of phosphate solutions after passing through ignited Pasteur-Chamberland filters*

| PORTION | $PO_4$ IN SOLUTION | |
|---|---|---|
| | Solution containing 0.2 p.p.m. $PO_4$ | Solution containing 0.5 p.p.m. $PO_4$ |
| | *p.p.m.* | *p.p.m.* |
| 1 | 0.380 | 0.600 |
| 2 | 0.140 | 0.408 |
| 3 | 0.150 | 0.412 |
| 4 | 0.152 | 0.404 |
| 5 | 0.170 | 0.408 |
| 6 | 0.164 | 0.408 |

results show that new filters do not absorb phosphorus to an appreciable extent. On the contrary the filters gave up a small amount of phosphorus to the more dilute phosphate solution. The low results for the first portion of the filtrate from the more concentrated solution are probably due to dilution by wash water held in the filter candle. Since these results show that new filters do not absorb phosphate, it is probable that soil colloids in the pores of the filters are the cause of phosphate absorption by used filters.

If the soil colloids in the pores of the filter are the cause of phosphate absorption any treatment of the filters that would tend to remove or destroy the

colloids would decrease the phosphate-absorbing power of the filter. The absorbing power of the colloid is probably due to the formation of iron and aluminum phosphates. The iron and aluminum of soil colloids is soluble in hot hydrochloric acid. Therefore, in order to determine whether or not the absorption of phosphate could be reduced or prevented, 4 used filters were treated with hot hydrochloric acid, washed, and then ignited in an electric furnace. The filters were placed in 250-cc. graduates, covered with 1:1 HCl, and put in a boiling water bath for 4 hours. After washing with distilled water the acid treatment was repeated, fresh acid being used. The filters were then washed with 1500 cc. of water and ignited in an electric furnace for 4 hours. This treatment should almost completely destroy the soil colloid held in the pores of the filter.

The filters were then washed with cold distilled water and phosphorus was determined in successive 100-cc. portions. The first 100 cc. contained 1.08 p.p.m. $PO_4$; the second portions, 0.126 p.p.m; the third, 0.095 p.p.m.; and the fourth, 0.08 p.p.m. $PO_4$ in solution. These figures are the averages for the 4 filters. After standing for several hours the filters were tested for absorption by filtering solutions containing 0.2 p.p.m. and 0.5 p.p.m. $PO_4$. Phosphorus was determined in successive 250-cc. portions with the results given in table 9.

The first portion of the filtered solutions contained more phosphorus than the original solution. This was doubtless due to phosphorus going into solution from the filter. The filters had stood about 4 hours after washing with distilled water. During this interval a large amount of phosphorus came into solution, probably by hydrolysis. The second and succeeding portions of the filtrate were lower in phosphorus than the original solution. Apparently the filters were absorbing phosphorus. However, the amount of absorption was probably somewhat less than it would have been with the same filters before treating with acid and igniting. Nevertheless the results show that it would not be advisable to use these filters for preparing soil extracts if a study of their phosphorus content is to be made.

## DISCUSSION

Pasteur-Chamberland filters have been extensively used in the preparation of soil extracts. In many instances the phosphorus content of the extract has been determined, but as far as the writer knows, the phosphate-absorbing power of the filter candle has never been taken into consideration. The results of several experiments, shown in tables 3 to 9, indicate that the phosphorus content of a solution may be considerably altered during filtration. The phosphorus content of the solution may be increased or decreased while passing through the filter candle. New Pasteur-Chamberland filters as well as those that have been used in preparing soil extracts contain considerable phosphorus that may be partially removed by washing with distilled water. If a soil extract of low phosphate content is filtered, its content of phosphorus may be increased by phosphate going into solution from the filter candle. Filters

that have been used in preparing soil extracts also have the ability to absorb phosphorus from a solution. The absorbing power is apparently due to soil colloids held in the pores of the filter candle. If a solution of rather high phosphorus content is filtered, part of the phosphorus will be absorbed by the filter candle. The amount absorbed may be sufficient to reduce the phosphate content of the solution to less than one-half of its original value. The error may be small or it may be rather large depending on the phosphorus content of the solution and the condition of the filter. In any case it would be difficult to make a satisfactory estimate of the error. Therefore, the use of Pasteur-Chamberland filters in the preparation of soil extracts for phosphorus studies should be discontinued.

This investigation did not include studies with solutions having a phosphate content greater than 1.0 p.p.m. $PO_4$. Therefore, the results of this study may not be applicable to results obtained with extracts containing several parts per million of phosphate. However it is probable that only a very few soils of the humid section of this country will yield extracts containing more than 1.0 p.p.m. $PO_4$.

## SUMMARY

1. The procedure used in the Coeruleo-molybdate method for determining phosphorus is given. Using this procedure one can determine phosphorus in solutions containing 0.02 p.p.m. $PO_4$.

2. The accuracy of the method is shown by comparing results obtained with it and with the standard volumetric method of determining phosphorus.

3. Pasteur-Chamberland filters that had been used in preparing soil extracts contained considerable phosphorus that is soluble in water or in solutions of low phosphate content.

4. Used filters absorbed considerable phosphorus from phosphate solutions of known concentration and from soil extracts containing 0.09 and 0.6 p.p.m. $PO_4$.

5. The absorption of phosphorus is apparently due largely to soil colloids held in the pores of the filter candle.

6. New Pasteur-Chamberland filters did not absorb phosphorus but contained considerable phosphorus soluble in water.

7. Treatment of used filters with strong hydrochloric acid and subsequent ignition in an electric furnace did not completely destroy the phosphate-absorbing power of used filters.

## ACKNOWLEDGMENT

The writer wishes to express his appreciation to Prof. M. J. Funchess for suggesting this investigation and for his valued suggestions and criticisms.

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# EFFECT OF STRAW ON ACCUMULATION OF NITRATES AND CROP GROWTH

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Received for publication April 27, 1925

The need for humus in our arid region soils is keenly felt. Straw is usually plentiful, and is the most natural source from which to fill this need, but very often when it is applied, ill effects of a more or less temporary nature are noticed.

The investigational work that has been done on the study of the effect of straw applications indicates that the carbon-nitrogen ratio is too wide, and that either a nitrogenous manure must be supplied with the straw, or that decomposition must proceed until the carbon-nitrogen ratio approaches 1:12, before crops receive a benefit from the manurial applications (11, 12). It has been found that straw decreases nitrification (1, 4, 10, 13, 15), but after a time increases crop growth (5, 15). Pfeiffer (8) noted harmful effects from the application of straw. Murray (7) and Waksman (14) suggest the idea that the straw is used as a source of carbon by bacteria, and whatever nitrogen is available, is utilized for the building of bacterial protoplasm, and, for the time being, is lost as available plant-food. Lyon, et al. (6) offer a similar explanation for the depressive influence of timothy on availability of nitrates. Collison and Conn (3) noted a decidedly depressive effect of straw applications on a subsequent crop. Collison (2) suggests that it may be due to certain organic compounds which are toxic to plants.

From the above brief review of literature, it is seen that while much has been learned of the influence of straw applications upon the productivity and bacterial activity of soils, more evidence is needed before we can draw sweeping conclusions for all conditions. In an attempt to throw a little more light upon this soil problem, a study was made of the influence of straw applications upon the accumulation of nitrates and crop growth, a representative Utah county soil classified by Sanchez (9) as "Jordan loam" being used.

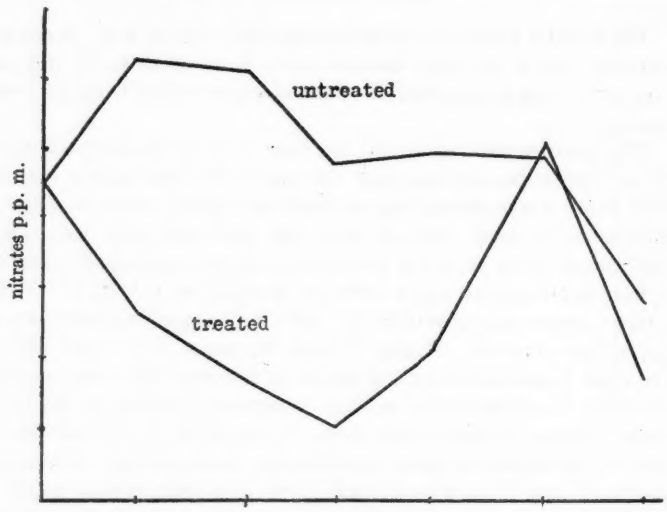
## PLAN OF EXPERIMENT

### *Experiment 1*

Gallon pots were filled with soil, and straw was applied at the rate of 5 tons per acre. The straw was cut into small pieces and thoroughly mixed with the soil which was kept at 25 per cent moisture content, dry basis, and incubated in

the greenhouse. Nitrates were determined at intervals by the phenol-disulphonic acid method. Each treatment was done in duplicate. The experiment was begun January 23, 1921 and ended April 18, 1922. The results of this experiment are presented in figure 1.

It will be noticed from figure 1 that the untreated pots show a downward trend in nitrate content, but the decrease is not nearly so great as that for straw-treated pots. An increase occurred in the nitrate content of the treated soil in both the March 18 and the April 4 analyses. This irregularity appears to be due to experimental error, although on both dates the duplicate pots checked




| | Jan. 23 | Feb. 3 | Feb. 20 | Mar. 4 | Mar. 18 | April 4 | April 18 |
|---|---|---|---|---|---|---|---|
| | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* |
| Untreated . | 226 | 310 | 302 | 233 | 240 | 236 | 136 |
| Treated ... | 226 | 131 | 83 | 53 | 104 | 250 | 84 |

FIG. 1. EFFECT OF STRAW APPLICATIONS ON NITRATE CONTENT OF THE SOILS AT DIFFERENT INTERVALS OF TIME

about as usual with nitrate determinations. The results agree with those of other experimenters in showing that straw was depressive in its effect on nitrification.

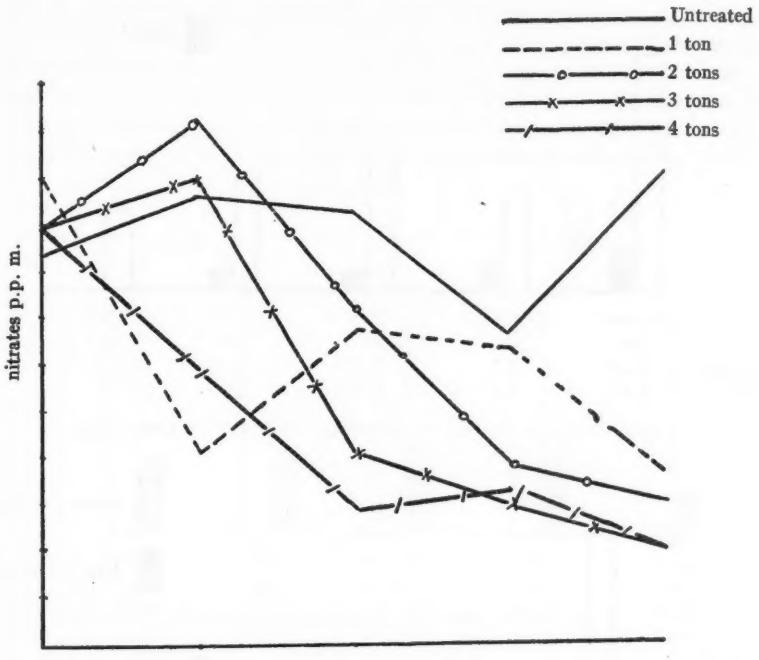
*Experiment 2*

"Would increasing the amounts of straw have a depressive effect on nitrification proportional to the increasing quantities?" With this question in mind the following experiment was carried out.

Gallon pots were filled with soil, and wheat straw was applied at the rate of 1 ton, 2 tons, 3 tons, and 4 tons per acre. Each straw application was run in

duplicate and one pot was used for a control. This made 9 pots in all. The treatment was similar to experiment 1. Nitrates were determined at intervals, with results as shown in figure 2.

While there are several irregularities noticeable in figure 2, especially in the analysis of January 10, the general trend is quite apparent. There is a decided



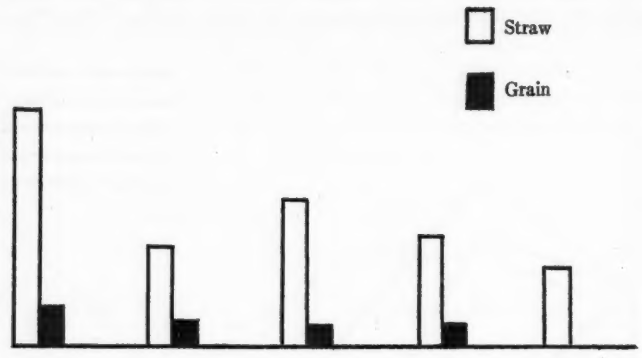

| | Dec. 1 | Jan. 10 | Feb. 28 | Mar. 19 | Mar. 24 |
|---|---|---|---|---|---|
| | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* |
| Untreated . . . | 84 | 96 | 88 | 66 | 100 |
| 1 ton straw. . | 100 | 42 | 67 | 68 | 35 |
| 2 tons straw. | 90 | 117 | 68 | 39 | 25 |
| 3 tons straw. | 90 | 100 | 41 | 30 | 20 |
| 4 tons straw. | 90 | 60 | 29 | 32 | 20 |

FIG. 2. EFFECT OF INCREASING AMOUNTS OF STRAW APPLICATION TO SOIL, ON THE NITRATE CONTENT AT VARIOUS PERIODS

trend downward in nitrate accumulations as a result of the application of straw, and the greater the amount of straw added, the greater the depression.

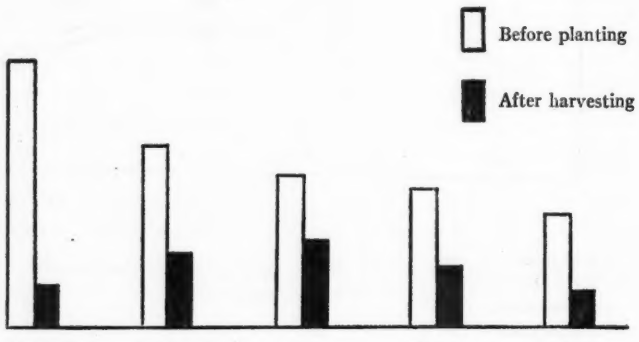
To see if the depressive effect noted for nitrates was also shown in crop growth, the pots treated in this experiment were planted to wheat, with results shown in figure 3. As there were only 2 pots for each treatment and 8 plants to a pot, the results for crop growth are not so regular as for nitrates, but the

general tread is quite apparent. There is a decided depression on crop yield as a result of the straw applications, and as the quantity of straw was increased, the crop yield was decreased.




| | Untreated | 1 ton | 2 tons | 3 tons | 4 tons |
|---|---|---|---|---|---|
| | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* |
| Straw.... | 6.85 | 2.7 | 4.1 | 3.1 | 2.2 |
| Grain.... | 1.05 | 0.5 | 0.4 | 0.45 | 0 |

FIG. 3. EFFECT OF INCREASING QUANTITIES OF STRAW ON CROP YIELD




| | Untreated | 1 ton | 2 tons | 3 tons | 4 tons |
|---|---|---|---|---|---|
| | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* | *p.p. m.* |
| Before planting.. | 29.4 | 19.6 | 16.4 | 15.25 | 12.65 |
| After harvesting. | 4.11 | 7.82 | 9.13 | 6.49 | 4.01 |

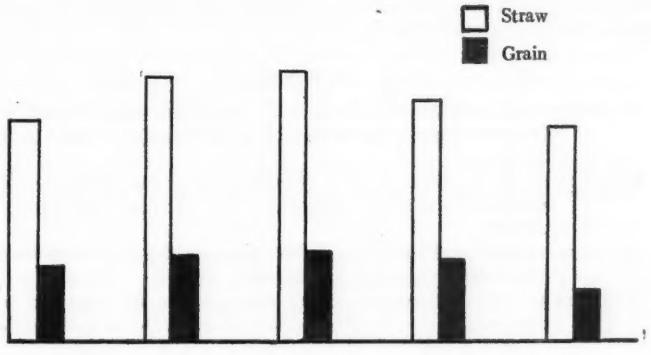
FIG. 4. AMOUNT OF NITRATES BEFORE PLANTING OF WHEAT, AND AFTER HARVESTING, ON SOILS THAT HAD BEEN TREATED WITH STRAW 12 MONTHS PREVIOUSLY

### *Experiment 3*

"Is this depressive effect of straw on nitrification and crop growth temporary or is its effect carried over to the following season?" was the next question

asked. To answer it, the pots and the soils in experiment 2 were used 12 months later, and the residual effect studied. In doing this the soils were pulverized, the nitrates determined, and another crop of wheat planted and harvested. After harvest the soils were analyzed again for nitrates. The results are shown in figure 4.

A glance at figure 4 shows that, before planting, the untreated pots contained nearly double the quantity of nitrates than any of the treated pots did, but during the growth of the crop, nitrification must have gone on more rapidly in the treated pots than in the untreated, for the crop grown (see fig. 5) was slightly greater in the treated pots than in the untreated with the exception of the 4-ton treatment; and yet after harvest there were more nitrates (with the exception again of the 4-ton treatment) in the treated than in the check.




| | Untreated | 1 ton | 2 tons | 3 tons | 4 tons |
|---|---|---|---|---|---|
| | *gms.* | *gms.* | *gms.* | *gms.* | *gms.* |
| Straw..... | 9.80 | 11.53 | 11.73 | 10.76 | 9.69 |
| Grain..... | 3.21 | 3.78 | 3.97 | 3.74 | 3.16 |

FIG. 5. EFFECT OF STRAW TREATMENT UPON GROWTH OF A WHEAT CROP AFTER A 12-MONTH INCUBATION PERIOD

This would indicate that the depressive effect of straw on nitrification and crop growth was only temporary, for during the second season the bad effects were practically overcome.

## SUMMARY OF RESULTS

Straw treatments in general caused a decline in the amount of nitrates accumulated in the soil.

The larger the quantity of straw added the greater the decline in soil nitrates. For the 4-month period the decline was quite regular, and in practically every instance less than the check.

The depressed crop yield tended to be proportionate to the nitrate decline.

After the straw had been allowed to decompose for a period of 12 months, its depressive effect upon nitrification was lessened, in fact nitrification went on more readily in the straw-treated pots.

## CONCLUSION

The results obtained from these experiments indicate that in the Jordan loam soil common to Utah County, wheat straw is detrimental to nitrification and to the growth of the crops, at least for a period. There is a desirable residual effect. The principal harm seems to be due to the retarded accumulation of nitrates in the soil.

The results of other investigators along this line, suggest the idea that the above is due to the fact that the nitrates in the presence of a large supply of carbonaceous matter are probably utilized by the increased numbers of bacteria that accumulate in the presence of a large supply of straw. After a period, a new supply of nitrogen accumulates in the soil, then further decomposition of straw ensues, the organic carbon is used up, and the by-products of decay together with a narrower carbon-nitrogen ratio create an environment more favorable to crop productivity.

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# LACK OF NODULE-FORMATION IN A SUBFAMILY OF THE LEGUMINOSAE

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Received for publication May 13, 1925

The prevalent idea that nodules caused by *Bacillus radicicola* Beij occur normally on the roots of all species of leguminous plants, is to a large extent true, but there are exceptions worthy of note occurring in the subfamily Caesalpinaceae. In the other two major subfamilies, the Mimosaceae and the Fabaceae, nodule-formation is found to be fairly constant if the proper organisms are in the soil; this is quite well established in view of the fact that most of our economic Leguminosae belong to the latter subfamily, but fewer data are available for members of the Mimosaceae.

The literature consulted concerning leguminous plants showed that the earlier authors gave the subject of root nodules more systematic attention than have the more recent investigators. This condition is probably due to an overshadowing of data of this character by other considerations, but it is nevertheless evident that such fundamental organs as nodules might properly have been given more consideration in plant descriptions.

Lachmann (6) in 1858 reported that nodules were not found on Cercis, and in 1888 and 1889 Buckhout (3) reported the lack of nodules on the monotypic genus Gymnocladus, *Cercis canadensis* L., and two other unnamed species of the subfamily. A few years later, Nobbe, Schmid, Hiltner and Hotter (9) report a failure to find nodules on *Gleditsia triacanthos* L. and express doubt as to the nodule-forming ability of the subfamily. Clos (4) in a review of the tubercle-forming plants reports the absence of nodules from the roots of young plants of *Cercis siliquastrum* and *Cassia barclayana* Sweet. Buchanan (2) on the other hand, states that nodules occur on *Cercis canadensis* L. and *Gleditsia triacanthos* L. Harrison and Barlow (5) report the lack of nodules on *Gleditsia*, *Gymnocladus*, and *Cercis canadensis* L.

Observations in the field and greenhouse and reports from coöperators have not produced any evidence to the effect that nodules are found on *Cercis canadensis* L., *Gleditsia triacanthos* L., or *Gymnocladus dioica* (L.) Koch.

It is stated by Beers (1) that the carob, *Ceratonia siliqua* L., adds nitrogen to the soil. Whether this statement is based on the assumption that nodules occur or that a mycorrhiza is present is not made clear. Specimens examined by me have shown no evidence of nodule formation, and a search of the literature has revealed nothing positive. Naudin (8) failed to find nodules on a

species of *Cassia* from Senegal and on *Caesalpinia brevifolia* Baill. from Chile. In the genus Caesalpinia, the species *exostemma* Moss. and Sesse, *melanocarpa* Griseb., and *pulcherrima* (L.) Sw. produced no nodules in greenhouse soil.

Numerous examinations in the field of *Cassia marylandica* L., *C. medsgeri* Shafer, *C. tora* L., and *C. occidentalis* L. by the writer, and of the latter two by J. C. Temple, Georgia Agricultural Experiment Station, have failed to reveal nodules. Greenhouse plants of the above named species, as well as of *C. corymbosa* Lam., *C. bicapsularis* L., *C. emarginata* L., *C. laevigata* Willd., *C. tomentosa* Wall., *C. artemisoides* Gaudlich, and of five unknown species gave the same result.

On the other hand, we find an exception in the genus Chamaechrista which is represented around Washington D. C., by the species *C. nictitans* L. (Moench) and *C. fasciculata* (Michx.) Greene. On both of these species nodules occur, and they have also been found on *C. simpsoni* Pollard from Florida.

A great many of the observations reported in this paper have been made on plants grown from seed or on plants obtained from the Office of Foreign Seed and Plant Introduction. It is realized that the observations are not so trustworthy as if they had been made on the plants in their native habitat, but they are confirmatory additions to the results hitherto reported.

In the subfamily Mimosaceae, examinations were made for nodules on twenty-four species of Acacia and of the closely related genera Albizzia and Prosopis. *Acacia baileyana* F. Muell. was the only species on the roots of which nodules were not formed. *Schrankia uncinata* Willd. and *Mimosa pudica* L., also of this subfamily, produced nodules.

The question naturally arises, why do these Leguminosae not produce nodules like others? If it is not a question of the presence or absence of the proper bacteria, and that point seems to be fairly well settled by these observations and those of investigators in various parts of the world herein reported, there must be a fundamental factor existing in the plants themselves which prevents nodule-formation. Nobbe and his co-workers (9) have suggested that the physical character of the root hairs of a few members of the Caesalpinaceae inhibits nodule-formation, and McDougall (7) comes to the same conclusion, namely that the thickwalled root hairs prevent the entrance of the bacteria. Whether this is the correct explanation of the problem remains for further work to decide. As a matter of fact, the problem extends beyond the Leguminosae, for it has not been determined why, with few exceptions, bacteria root nodules do not occur on non-leguminous plants, or, stated in another way, why they are peculiar to leguminous plants only.

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# COLLOIDAL BEHAVIOR OF SOILS AND SOIL FERTILITY: I. SUCTION FORCE OF SOILS AS AN INDEX OF THEIR COLLOID CONTENT[1]

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Received for publication May 15, 1925

The vital significance of the soil colloids in fertility processes will be pointed out in another paper of this series. It will be shown that a more rational understanding of the various phenomena connected with the problem of colloids in soil will help to place soil science on a sound scientific basis.

One of the chief concerns of the soil investigator in connection with the study of colloids is a method for determining the colloid content of the soil. Various attempts have been made to estimate the colloidal fraction of the soil directly or indirectly. Wache (13) in his contribution on the determination and appreciation of colloids in soil, reviews historically and critically the methods in vogue. The studies on the estimation of colloids like those from the Bureau of Soils (1, 2, 6, 7, 11) and others, like those by Bouyoucos (3, 4), which were reported after the publication of Wache's paper, may be well fitted into the classification of Wache. The method of Bouyoucos is the heat of wetting which is also the method of Anderson (1). The latter claims that

> This (*heat of wetting*)[2] method is subject to some inaccuracy, due to failure to isolate a sample representative of the whole of the colloid and due to alteration of the colloids through the process of extraction. On the whole, the heats of wetting of soil and colloid probably indicate the colloid content of a soil about as accurately as adsorption determination.

At this point it is well to recall that the heat of wetting method has been used by Mitscherlich (10), not to determine the colloids as was done by Bouyoucos or Anderson, but to determine the hygroscopicity which is a function of the soil surface, chiefly of the colloid content. It has been recognized by Mitscherlich that with certain limitations the hygroscopicity is proportional to the surface, and since colloids are known for their surface a determination of the hygroscopicity may serve as a measure of the colloid content of the soil. Wache (13) points out that this method cannot be used as a measure of the colloid content of the soil for the principal reason that the hygroscopicity varies with the variations in the state of the colloids (*reversibility and irreversi-*

[1] Paper no. 231 of the Journal Series, New Jersey Agricultural Experiment Stations, Department of Soil Chemistry and Bacteriology.

[2] Parenthetical insertion by authors.

*bility*).[3] In our own minds it seems that this point has been overlooked entirely by all the investigators. The soil colloids from the nature of the dynamics of soil processes cannot be a constant, hence the statement in the next paragraph with reference to our method.

The authors suggest another method for the estimation of soil colloids; it is an indirect method and is based on the property of colloids to adsorb water. *It would be better to say that the method gives an idea of the quantity of colloids in the soil as it is manifested by the colloid reaction properties of the soil, at the moment and condition of the experiment.* The method under consideration has not as yet been fully tested out in the laboratory of the New Jersey Agricultural Experiment Station, although substantial progress has been made. This communication is presented with the hope that other workers in the field will try it out, as in our estimation it has well-founded possibilities and is not cumbersome, even less so than the heat of wetting method.

The method proposed is an outgrowth of the work of Kornev (8, 9). Since this work was reported in a Russian journal it is felt that a complete description of it would be helpful. Kornev (8) points out that the suction force of soils has not been investigated in a quantitative way. He worked out a simple method whereby this force may be measured quantitatively with great accuracy. The apparatus used is based on the principle that porous substances, like slightly burned clay, are able to admit or diffuse water through their pores and at the same time not permit any air (unless very high pressure is applied) to enter or pass out. When a porous material, such as a Pasteur clay filter or filter balloon (the latter has been used by the authors in most cases; the accompanying diagram is self evident) is filled with water, connected with a mercury manometer and then immersed in a column of soil, water will begin immediately to stream from the porous container into the layer of soil in direct contact with the container. When the suction force begins to act, a negative pressure is produced in the porous container and is recorded on the manometer. When the system has come to an equilibrium, i.e , when there is no more suction produced, the rise of the mercury column is a direct measure of this force. The suction force ceases when the layer of soil in contact with the porous container has become saturated. Corrections are to be made for (*a*) the variations in barometric pressure, (*b*) the volume of air present in the apparatus, and (*c*) the height of the water column in the container, since this column exerts a pressure of its own, forcing out water. Temperature changes are to be avoided of course. Kornev (8) suggests as a practical application of the suction force of soils the utilization of this force in irrigation works. The suction force, however, affords a very convenient method for the study of various other soil problems.

Table 1 shows some of the data on the suction force of soils.

[3] Parenthetical insertion by authors.

The outstanding feature of the data in table 1 is, that as the soil is enriched in fine particles beginning from sand to clay, the suction force increases. In other words with the increase of the colloidal fraction the suction force is increased. Pure quartz sand which has been washed has practically no suction force. Additions of colloids to the sand increase the suction force. The significance of this force cannot be over-estimated, as it represents a clear-cut physico-chemical property, inasmuch as the force is chiefly of a capillary and molecular character and therefore governed by the surface energy so intimately connected with colloidal behavior.

A very interesting feature demonstrated by the suction force is the comparative response of the surface and subsoil materials of the Portsmouth loam (436 and 437A) and silt loam (466 and 467). It will be noted that the surface

TABLE 1

*Suction force of various types of soil*

| SOIL NUMBER | TYPE OF SOIL | SUCTION FORCE: RISE OF MERCURY COLUMN* |
|---|---|---|
| | | *cm.* |
| 0 | Sand (sea) | 1.2 |
| 428 | Lakewood sand | 6.0 |
| 331 | Sassafras sand, slightly loamy | 13.0 |
| 417 | Collington sandy loam | 9.8 |
| 353 | Sassafras sandy loam | 16.6 |
| 446 | Shrewsbury heavy sandy loam | 21.1 |
| 436 | Portsmouth loam | 28.0 |
| 437A | Portsmouth loam: subsoil | 26.7 |
| 165 | Layton loam | 29.2 |
| 463 | Elkton loam: organic matter | 29.6 |
| 466 | Silt loam | 32.7 |
| 467 | Subsoil of 466 | 32.3 |
| | Alloway clay | 40.0 |

*The figures given are averages of several determinations.

soil exhibits a higher suction force. An examination of the variation in the physical make-up of the respective soil layers showed that the surface soils are rich in organic matter. The phenomenon is suggestive and the explanation must thus be sought in the organic matter. Apparently the organic colloids with their high degree of dispersion possess great surface energy and hence exert a greater suction force. While no definite statement may be made as to the significance of the observed variation in suction force, this much is worthy of supposition: if it is true that the difference in suction force exhibited by the surface and subsoils is due to the organic colloids, then the method might offer a convenient index of the proportion of chemically active organic matter, which in reality means the soil organic colloids. The differentiation of active (colloidal) and inactive organic matter has been analyzed by Sokolovski (12).

Since the suction force is an expression of the surface energy of the colloidal fraction of the soil, then the ratio:

$$\frac{\text{the suction force of the soil}}{\text{the suction force of the colloids}} \times 100$$

ought to give the percentage of colloids in the soil. The inadequate equipment for the separation of the colloids makes it, for the present, impossible to test out fully the method suggested. By separating out some of the colloids by fractionation of the soil, it was found that the method may be used to good advantage to estimate the colloid content of the soil. More data is being collected to establish the point.

The problem was also attacked from another angle. Quartz sand which manifests but little suction force was mixed with highly colloidal substances, such as bentonite, and the suction force measured. Table 2 gives the results of several determinations.

TABLE 2

*Suction force of sand with and without bentonite*

| LABORATORY NUMBER OF SAMPLE | TREATMENT | SUCTION FORCE: RISE OF MERCURY COLUMN |
|---|---|---|
| | | *cm.* |
| 1 | Quartz sand | 1.2 |
| 2 | Sand + 5 per cent bentonite | 6.8 |
| 3 | Sand + 10 per cent bentonite | 9.8 |
| 4 | Sand + 20 per cent bentonite | 16.0 |
| 5 | 100 per cent bentonite | 24.0 |

It may be seen readily that with the increase in colloid content the suction force increases. On account of the variation in the size of the quartz particles there is difficulty in covering them and there is no well-defined proportionality factor which should show the relation of the increase in colloid content.

Incomplete experiments with pure clay colloids mixed in varying proportions with different soils, starting with almost a pure sand, seem to indicate that there *is* a correlation between the amount of colloids present and the suction force. It is hoped that by applying statistical methods there will be a chance to determine directly the amount of colloids present without the tedious process of separating them out. From some data on hand it is hoped that graphic presentation of such data may aid greatly in establishing some important colloidal properties of the soil.

That fertilizers do exert a profound influence on the soil colloids is known. Ehrenberg (5) justly remarks that this is to be expected, since fertilizers consist chiefly of substances with highly reactive properties. Still, outside of the effect of lime, very few quantitative studies have been made on the effect of various fertilizer treatments on soil colloids. There has been a great number of investigations on the effect of fertilizers on the physical properties

of the soil; in a great measure these effects could be traced chiefly in the colloid fraction of the soil. The reason for such a lack of interest in regard to fertilizer treatment and colloid behavior may be traced in the lack of methods for the study of soil colloids. The suction force affords a very convenient method for the expression of colloid reactions.

This paper presents only one experiment showing the effects of fertilizer treatment on soil colloids. Table 3 gives the outline and the results of the experiment.

No sweeping conclusions may be made from the data presented, but one outstanding feature is of interest. The presence of lime in these soils depresses the suction force. It is reasonable to suppose that the Ca ions as they are gradually liberated from the slowly soluble limestone coagulate the colloids, decreasing their surface energy. There may be a condition when an excess of

TABLE 3

*Suction force of soils variously treated*

| SOIL NUMBER | TREATMENT | SUCTION FORCE: RISE OF MERCURY COLUMN |
|---|---|---|
| | | *cm.* |
| 1 | None | 28.1 |
| $1_1$ | Limestone, 2000 to 4000 pounds every 5 years | 25.0 |
| 2 | $(NH_4)_2SO_4$: equivalent to 320 pounds of $NaNO_3$ in terms of N + minerals* | 24.6 |
| $2_1$ | Same as 2 + lime as in $1_1$ | 21.5 |
| 3 | $Ca(NO_3)_2$: equivalent to 320 pounds of $NaNO_3$ in terms of N + minerals | 28.0 |
| $3_1$ | Same as 3 + lime as in $1_1$ | 25.6 |
| 4 | $CaCN_2$: equivalent to 320 pounds of $NaNO_3$ in terms of N + minerals | 21.9 |
| $4_1$ | Same as 4 + lime as in $1_1$ | 18.1 |

* Under minerals are understood 400 pounds of acid phosphate and 200 pounds of muriate of potash per acre.

Ca ions in the presence of K or Na ions will cause the formation of NaOH or KOH and eventually $K_2CO_3$ or $Na_2CO_3$. These reagents are known for their effective peptization action. Under such conditions the degree of dispersion of the soil colloids is increased and the suction force increased proportionately; it is for this reason that alkali soil colloids are highly dispersed.

More data on this subject will be presented in a forthcoming paper of this series.

## SUMMARY

The method of Kornev (8) for the study of the suction force of the soil is presented and analyzed, data illustrating the method being given.

The suction force of the soil, being an expression of the molecular and capillary forces, may be used as an index of the colloid content of the soil; the forces which govern the suction force are therefore to be looked for in the soil

colloids, which by virtue of their surface energy, possess and exhibit the properties which produce this suction force.

Variations in the suction force of like soils with a variable organic matter content seem to indicate the plausibility of using the expression of this force in determining the amount of organic colloids in soils.

The ratio of the suction force of any soil to the suction force of the isolated colloids of the same soil multiplied by 100 may serve as a convenient index of the quantity of colloids present in the soil under the conditions and time of the experiment.

There is an indication that graphic presentation of data on the suction force of various types of soils may serve as a means of establishing not only the quantitative relationships of the colloids in the soil, but also many other points in connection with the colloidal behavior of soils. Statistical methods may be used in this connection.

The effect of fertilizers and lime on the colloidal behavior of soils as expressed by the suction force of the soil was studied and the rôle of some cations analyzed.

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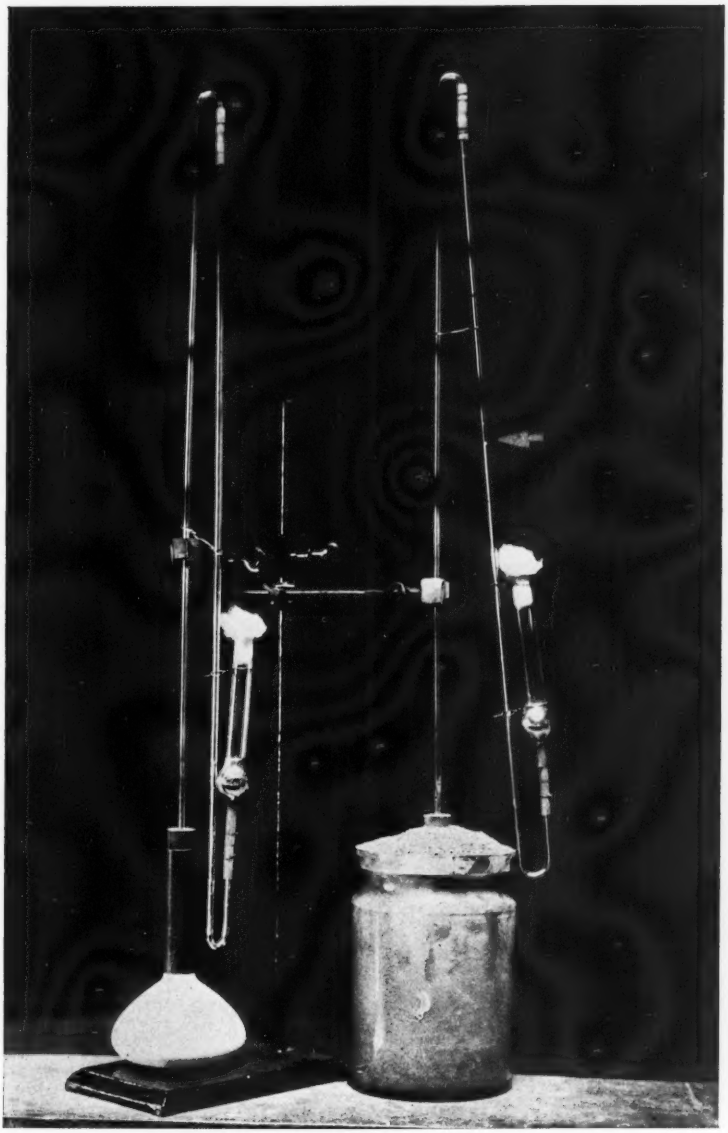
## PLATE 1

ARRANGEMENT OF SUCTION APPARATUS

Arrow point to the right indicates rise of mercury column



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# DISTRIBUTION OF NITROGEN IN THE PODSOL PROFILE

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Received for publication, May 15, 1925

Podsol soils, confined in this country largely to northern New England and the northern part of the Great Lakes region,[1] are distinguished in the field by the following profile:

The first horizon, $A_o$, consists of slightly decomposed forest débris and moss, called "raw humus" by German investigators. Directly below the raw humus, is a layer of mineral soil known as horizon A. This may be gray throughout, or the upper part may be dark in color owing to the presence of considerable organic matter. In the latter case the A horizon is subdivided into $A_1$, the darker layer, and $A_2$, the gray layer. The B horizon below A, is of heavier texture and may even contain a hardpan. The upper part of B, horizon $B_1$, is brownish and the lower part, horizon $B_2$, is brighter in color, either reddish or yellowish. The C horizon below B is the parent soil-forming material, which in this country is usually of glacial origin.

In the laboratory, the chief physical distinction between the horizons lies in the heavier texture of horizon B. The chemical characteristics of the profile that have been chiefly emphasized are the accumulated organic matter on the true soil surface, and the larger quantities of humus, iron oxide and alumina in the B horizon than in the A or C horizons.

Very little has been published on the distribution of nitrogen in the podsol profile.[2] This is doubtless because in the early chemical work on the profile, determinations were stressed which would explain visible field distinctions.

An examination made of a series of soils from Cherry Mountain, New Hampshire, submitted by the Northwestern Forest Experiment Station, indicates that there is a characteristic distribution of nitrogen in this profile. The samples were identified by Dr. C. F. Marbut as being typical podsol horizons. The nitrogen content, acidity and moisture equivalent of the samples are shown in table 1.

The four samples designated as $A_1$ in profiles I to V were very low in minerals, the loss on ignition being 72 per cent, 83 per cent, 77 per cent, 51 per cent and 84 per cent, respectively. With such low contents of mineral matter

[1] Personal statement of Dr. C. F. Marbut. See also map in article by Marbut (4, p. 132).

[2] In the analysis of podsol profiles reported by Glinka (3) and by Frosterus (2), nitrogen was not determined. McCool, Veatch and Spurway (5) give the nitrogen contents of many Michigan soil profiles, a few of which are evidently typical podsols.

it is doubtful whether they should be regarded as $A_1$ horizons. Possibly all except $A_1$ of profile IV would be more properly classified as the lower part of the $A_0$ horizon. They were not representative of the whole $A_0$ horizon, since they contained none of the coarser organic debris. The C samples were evidently only the upper part of the C horizon or possibly even the lower part of $B_2$. Deeper samples would be needed to decide this question.

In all five profiles the distribution of nitrogen is marked and distinctive. The nitrogen content is, of course, very high in the organic layer; it is low in

TABLE 1

*Nitrogen, acidity and moisture equivalent of podsol horizons*

| NUMBER OF PROFILE | DEPTH OF SAMPLE | HORIZON | COLOR OF HORIZON | NITROGEN (N) CONTENT | ACIDITY | MOISTURE EQUIVALENT |
|---|---|---|---|---|---|---|
| | *inches* | | | *per cent* | *pH* | |
| I | 1 | $A_1$ | Dark brown | 1.622 | 4.3 | 101.7 |
| | 4 | $A_2$ | Light gray | 0.055 | 4.4 | 22.4 |
| | 5 | B | Brown | 0.426 | 5.0 | 79.6 |
| | 7 | C | Light yellow brown | 0.205 | 4.6 | 26.5 |
| II | 1 | $A_1$ | Dark brown | 1.995 | 4.4 | 156.0 |
| | 4 | $A_2$ | Gray | 0.133 | 4.4 | 24.5 |
| | 5–8 | B | Brown | 0.422 | 4.6 | 62.7 |
| | 8–13 | C | Light yellow brown | 0.177 | 4.6 | 33.8 |
| III | 1 | $A_1$ | Dark brown | 1.755 | 4.6 | 147.6 |
| | 3 | $A_2$ | Gray | 0.131 | 4.4 | 24.9 |
| | 6 | B | Brown | 0.289 | 4.6 | 72.2 |
| | 10 | C | Dark gray brown | 0.219 | 4.6 | 48.1 |
| IV | 1½ | $A_1$ | Dark brown | 1.400 | 4.6 | 190.8 |
| | 6 | $A_2$ | Light gray | 0.051 | 4.4 | 11.2 |
| | 9 | B | Brown | 0.279 | 4.8 | 33.2 |
| | 12 | C | Light yellow brown | 0.055 | 4.8 | 13.1 |
| V | 2 | $A_1$ | Dark brown | 1.530 | 5.4 | 148.7 |
| | 3 | $A_2$ | Gray brown | 0.123 | 4.4 | 26.8 |
| | 12 | B | Brown | 0.325 | 5.0 | 65.7 |

the leached $A_2$ horizon, increases markedly in the B horizon, and falls off sharply in upper C to a quantity somewhat greater than that in the $A_2$ horizon.

The acidity of the different horizons varies little in degree, although there is possibly a tendency for the leached $A_2$ horizon to give a slightly lower pH value than the others.

Doubtless many podsol profiles have a less marked distribution of nitrogen than these from New Hampshire. Although these profiles were not deeply developed, they had the appearance of pronounced examples. This is also indicated by the moisture equivalent determinations, which give an approximate idea of the relative contents of very fine material in the $A_2$, B and C

horizons.[3] It is evident that there is much more fine material in B, than in $A_2$ or C; hence the profile was markedly developed physically.

It is of course to be expected that chemical differences in the horizons would parallel physical differences to some extent. As a matter of fact, there is a rough correspondence between the variations in nitrogen and the variations in moisture equivalent in the different horizons. A similar correspondence between nitrogen and heat of wetting determinations is apparent in the data reported by McCool, Veatch and Spurway (5).[4] The profiles reported by them which show a "podsol" distribution of nitrogen show a similar distribution of the heat of wetting. Likewise, where the heat of wetting indicates no concentration of fine material in the B horizon the nitrogen decreases regularly downward.

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[3] It has been shown by Middleton (6) that the clay separate is the soil fraction of most importance in determining the moisture equivalent.

[4] The heat of wetting reflects the fine material in the soil even more than the moisture equivalent determination. According to Anderson (1), the heat of wetting of a soil is due almost exclusively to the colloidal material, and different soil colloids show different heats of wetting. An increase in the heat of wetting in part of a profile, therefore, indicates an increase in colloid content or a change in kind of colloid.